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## PATENT APPLICATION TRANSMITTAL LETTER

To the Assistant Commissioner for Patents:

Transmitted herewith for filing under 35 U.S.C. 111 and 37 C.F.R. §1.53 is the patent application of: Gulilat Gebeyehu and Joel A. Jessee

entitled: Novel Reagents for Intracellular Delivery of Macromolecules

Enclosed are:

- ☒ 62 pages of written description, claims and abstract.
- ☒ 0 sheets of drawings.
- ☒ an assignment of the invention to Life Technologies, Inc.
- ☒ a true copy of the declaration of the inventors and power of attorney filed in USSN 08/195,866.
- ☐ a certified copy of a \_\_\_\_\_ application.
- ☐ associate power of attorney.
- ☐ a verified statement to establish small entity status under 37 CFR §1.9 and §1.27.
- ☐ information disclosure statement.
- ☐ preliminary amendment
- ☐ other:

09/326106  
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### CLAIMS AS FILED

	Number Filed	Number Extra	Rate	Fee
BASIC FEE			\$760	\$760
TOTAL CLAIMS	30-20=	10	x \$18	\$180
INDEPENDENT CLAIMS	3-3=	0	x \$78	
MULTIPLE DEPENDENT CLAIM PRESENT			x \$260	

\* Number extra must be zero or larger **TOTAL \$940**

If applicant has small entity status under 37 C.F.R. 1.9 and 1.27, then divide total fee by 2, and enter amount here.

**SMALL ENTITY TOTAL**

\$

$$\overline{X}$$
X


— Charge the amount of \$\_\_\_\_\_ as filing fee.

X Charge any additional filing fees required under 37 CFR 1.16 and 1.17.

— Allowance, pursuant to 37 CFR 1.311(b).

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NOVEL REAGENTS FOR INTRACELLULAR  
DELIVERY OF MACROMOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent  
Application Serial No. 08/195,866, filed February 11, 1994, which  
is incorporated by reference in its entirety herein to the extent  
not inconsistent herewith.

FIELD OF THE INVENTION

Cationic lipid compounds are disclosed, having utility in  
lipid aggregates for delivery of macromolecules and other  
compounds into cells. Also disclosed are compositions of  
cationic lipids and viral components or non-viral fusagenic  
compounds useful for enhancing transfection.

BACKGROUND OF THE INVENTION

Lipid aggregates such as liposomes have been found to be  
useful as agents for delivery to introduce macromolecules, such  
as DNA, RNA, protein, and small chemical compounds such as  
pharmaceuticals, to cells. In particular, lipid aggregates  
comprising cationic lipid components have been shown to be  
especially effective for delivering anionic molecules to cells.  
In part, the effectiveness of cationic lipids is thought to  
result from enhanced affinity for cells, many of which bear a net  
negative charge. Also in part, the net positive charge on lipid  
aggregates comprising a cationic lipid enables the aggregate to

bind polyanions, such as nucleic acids. Lipid aggregates containing DNA are known to be effective agents for efficient transfection of target cells.

5 The structure of various types of lipid aggregates varies, depending on composition and method of forming the aggregate. Such aggregates include liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, having particle sizes in the nanometer to micrometer range. Methods of making lipid aggregates are by now well-known in the art. The main drawback to use of conventional phospholipid-containing liposomes for delivery is that the material to be delivered must be encapsulated and the liposome composition has a net negative charge which is not attracted to the negatively charged cell surface. By combining cationic lipid compounds with a phospholipid, positively charged vesicles and other types of lipid aggregates can bind DNA, which is negatively charged, can be taken up by target cells, and can transfect target cells. (Felgner, P.L. et al. (1987) Proc. Natl. Acad. Sci. USA 84:7413-7417; Eppstein, D. et al., U.S. Patent 4,897,355.)

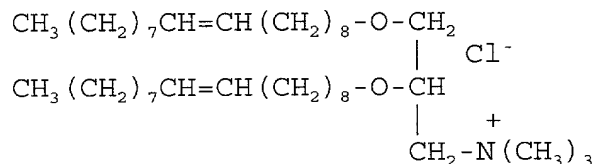
Cationic lipids useful for transfection and intracellular delivery of macromolecules generally contain the following four structural elements:

25 Lipophilic - Linker - Spacer - Cationic  
group bond arm group

30 The lipophilic group is a hydrophobic moiety which facilitates the insertion of the cationic amphiphile into the membranes of the cell or liposome. The lipophilic group serves as an anchor for the cationic group (usually ammonium) which is positively charged at neutral Ph, to attach to the surface of the cell or liposome. The spacer arm is typically a hydrophilic, 2

to 15-atom moiety which connects the cationic group to the lipophilic group via the linker bond. The linker bond is either an ether, ester, amide or other hydrolyzable bond.

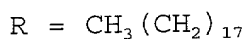
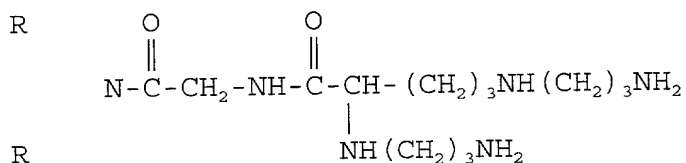
A well-known cationic lipid disclosed in the prior art is N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). The structure of DOTMA is:



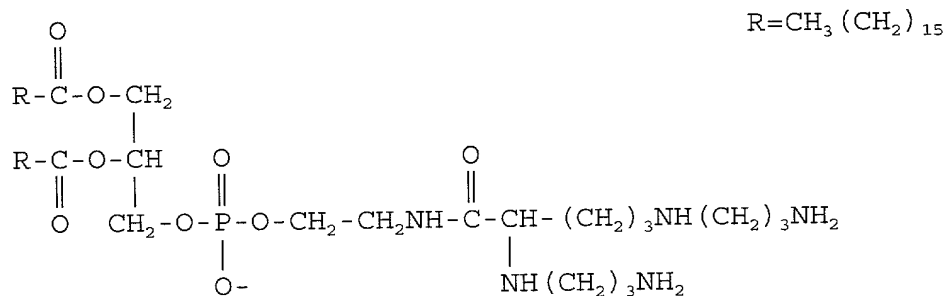
DOTMA by itself or in 1:1 combination with dioleoylphosphatidylethanolamine (DOPE) is formulated into liposomes using standard techniques. Felgner, et al. supra demonstrated that such liposomes provided efficient delivery of nucleic acids to some types of cells. A DOTMA:DOPE (1:1) formulation is sold under the trade name LIPOFECTIN (Gibco/BRL: Life Technologies, Inc., Gaithersburg, MD). Another commercially available cationic lipid is 1,2-bis(oleoyloxy)-3-(trimethylammonia) propane (DOTAP), which differs from DOTMA only in that the oleoyl moieties are linked via ester, rather than ether bonds to the propylamine. DOTAP is believed to be more readily degraded by target cells. A related group of prior art compounds differ from DOTMA and DOTAP in that one of the methyl groups of the trimethylammonium group is replaced by a hydroxyethyl group. Compounds of this type are similar to the Rosenthal Inhibitor (RI) of phospholipase A (Rosenthal, A.F. and Geyer, R.P. (1960) J. Biol. Chem. 235:2202-2206) which has stearoyl esters linked to the propylamine core. The dioleoyl analogs of RI are commonly abbreviated as DORI-ether and DORI-ester, depending on the linkage of the fatty acid moieties to the propylamine core. The hydroxy group can be used as a site for further

functionalization, for example by esterification to carboxyspermine.

Another class of prior art compounds has been disclosed by Behr et al. (1989) Proc. Natl. Acad. Sci. USA 86:6982-6986; EPO publication 0 394 111 (Oct. 24, 1990), in which carboxyspermine has been conjugated to two types of lipids. The structures of 5-carboxyspermylglycine dioctadecylamide (DOGS) is:



The structure of dipalmitoylphosphatidylethanolamine 5-carboxyspermylamide (DPPES) is:



Both DOGS and DPPES have been used to coat plasmids, forming a lipid aggregate complex that provides efficient transfection. The compounds are claimed to be more efficient and less toxic than DOTMA for transfection of some cell lines. DOGS is available commercially as TRANSFECTAM<sup>TM</sup> (Promega, Madison, WI).

A cationic cholesterol derivative (DC-Chol) has been synthesized and formulated into liposomes in combination with

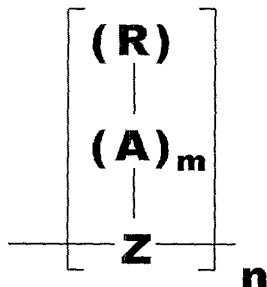


respect to several of the foregoing attributes. Compositions of the invention comprising cationic lipids in combination with viral components or non-viral fusagenic compounds are particularly useful for enhancing the efficiency of transfection and/or the range of delivery capabilities.

#### SUMMARY OF THE INVENTION

The present invention provides novel cationic lipids according to the general formula:

FORMULA I



In the general formula (I),

R is  $R_A$  or  $R_B$ , where  $R_A$  is  $C_{1-23}$  alkyl or alkenyl and  $R_B$  is a steroid selected from the group consisting of stigmasterol, ergosterol and cholic acid;

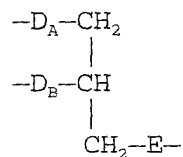
n is an integer ranging from 1 to about 2,000 where n is greater than 1 only when Z is  $Z_{16}$  or  $Z_{17}$ ;

m is 0 or 1, where m is 0 when Z is  $Z_{16}$  or  $Z_{17}$  and m is 1 when Z is  $Z_1$ - $Z_{15}$  or  $Z_{18}$ ;

A is selected from any of  $A_1$ - $A_3$  where



A<sub>1</sub> is



where D<sub>A</sub> and D<sub>B</sub>, independently of one another, are selected from the group consisting of D<sub>1</sub>-D<sub>3</sub> where:

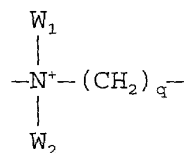
D<sub>1</sub> is -Y<sub>1</sub>-CO-Y<sub>2</sub>-, where Y<sub>1</sub> and Y<sub>2</sub>, independently of one another, are O and N, wherein at least one of Y<sub>1</sub> and Y<sub>2</sub> is N;

D<sub>2</sub> is -CH=CH-O-;

D<sub>3</sub> is -O- or -CO<sub>2</sub>-; and

wherein E is selected from the group consisting of E<sub>1</sub>-E<sub>3</sub> where:

E<sub>1</sub> is

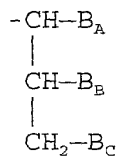


, where W<sub>1</sub> and W<sub>2</sub>, independently of one another, are C<sub>1-24</sub> alkyl, alkenyl or aryl; q is 1 to 6;

E<sub>2</sub> is -PO<sub>4</sub><sup>-</sup>-(CH<sub>2</sub>)<sub>n</sub>-NH-, where n is 2-6 with n of 2 being preferred;

E<sub>3</sub> is -(PO<sub>4</sub><sup>-</sup>)<sub>r</sub>-[inositol]-NH-, where r is 1 or 2;

A<sub>2</sub> is



where  $B_A-B_C$ , independently of one another, are selected from the group consisting of the following groups  $B_1-B_4$ , wherein one of  $B_A-B_C$  is  $B_1$ , one of  $B_A-B_C$  is  $B_2$ , and one of  $B_A-B_C$  is  $B_3$  or  $B_4$ ;

5

$B_1$  is  $-OH$ ;

$B_2$  is  $-NH-R$ , where  $R$  is  $C_{1-23}$  alkyl, alkenyl or acyl;

$B_3$  is  $-O-Z$  or  $-NH-Z$ ; and

10

$B_4$  is  $-PO_4^--(CH_2)_n-NH-Z$ , where  $n$  is 2-6 with  $n$  of 2 being preferred;

$A_3$  is  $-NH-CH_2-$  or  $-CO-N-R_1-$ , where  $A$  is  $-NH-CH_2-$  when  $R$  is cholic acid and  $A$  is  $-CO-N-R_1-$  when  $R$  is stigmasterol or ergosterol;

15

where  $R_1$  is an alkyl, alkenyl, alkynyl, alkoxy, acyl or alkylthio having from 1 to about 24 carbon atoms; and

where  $Z$  is selected from the group consisting of  $Z_1-Z_{17}$ , where

20

$Z_1$  is  $H$  except where  $W_1$  and  $W_2$  are methyl;

$Z_2$  is  $-(CH_2)_n-X$ , where  $n$  is 1-24 and  $X$  is selected from the group consisting of  $Br$ ,  $Cl$ ,  $I$  and  $F$ ;

$Z_3$  is  $-(CH_2)_n-NH_2$ ,  $n = 1-24$ ;

$Z_4$  is  $-CH_2-NH-(CH_2)_3-NH-(CH_2)_4-NH_2$ ;

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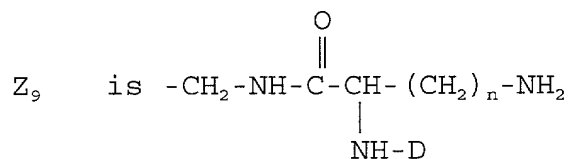
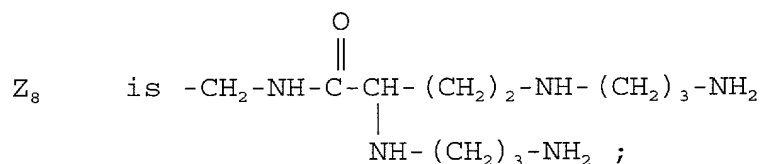
$Z_5$  is  $-CH_2-NH-(CH_2)_3-NH-(CH_2)_3-NH-(CH_2)_3-NH_2$ ;

$Z_6$  is  $-CH_2-NH-(CH_2)_n-NH_2$ ,  $n = 2-24$ ;

30

$Z_7$  is  $-L-X$  where  $L$  is selected from the group consisting of branched or straight chain alkyl, alkenyl, cycloalkyl, aryl, alkoxy, thioalkyl and thioether groups having from 1 to about 24 carbon atoms, and  $X$  is selected from the group consisting of  $Br$ ,  $Cl$ ,  $I$ ,  $F$ ,  $NH_2$  and  $[(NH_2)-(CH_2)_n]_m$  where  $n$  is 2-24 and  $m$  is 1-24;

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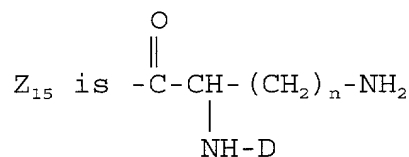
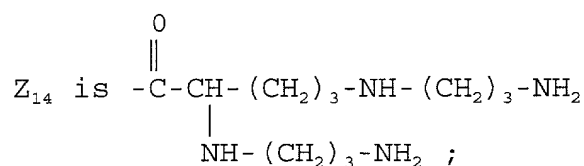
where  $n = 1-24$ ,  $D$  is  $H$  or other groups attached by amide or alkyl amino groups;

$Z_{10}$  is a reporter molecule;

$Z_{11}$  is a protein, peptide or polypeptide;

$Z_{12}$  is a polysaccharide;

$Z_{13}$  is an amine or halide reactive group;



$n = 1-24$ ,  $D$  is  $H$  or other groups attached by amide or alkyl amino groups;

$Z_{16}$  is an amino acid;

$Z_{17}$  is a monosaccharide; and

$Z_{18}$  is a nucleic acid binding substance.

Compounds of the invention are useful, either alone or in combination with other lipid aggregate-forming components (e.g., DOPE, DOSPA, DOTMA or cholesterol) for formulation into liposomes or other lipid aggregates. Such aggregates are cationic, able to form stable complexes with anionic macromolecules, such as nucleic acids. The lipid aggregate macromolecular complex

interacts with cells making the macromolecule available for absorption and uptake by the cell. The halogenated compounds of the invention are also especially useful as intermediates for chemically coupling the cationic lipid to reporter molecules, proteins, polypeptides, antibodies, polysaccharides and the like to permit targeted delivery, quantitative assessment of targeting, greater efficiency of delivery and enhanced range of delivery capabilities.

Compounds of the invention comprising a nucleic acid binding substance are particularly useful in applications requiring targeted delivery to DNA or RNA. Nucleic acid binding substances include, without limitation, histones, protamines, polycationic peptides, intercalators, polyamines, and nucleic acid binding proteins or domains thereof. Useful nucleic acid binding proteins comprise DNA-binding motifs such as a helix-turn-helix DNA binding motif or a zinc finger. Zinc fingers occur in a variety of eukaryotic transcription factors including Spl, estrogen, and glucocorticoid receptors, several Drosophila developmental regulators, Xenopus Xfin protein, the E. coli UvrA protein, and certain retroviral nucleic acid binding proteins. Compounds of the invention comprising protein, peptide and polypeptide substituents are also useful for binding a variety of other substances. For example, lectins such as concanavalin A and wheat germ agglutinin are useful for binding sugars and  $\beta$ -N-acetylmuranlic acid and  $\alpha$ -N-acetylneuraminic acid, respectively.

Compounds of the invention can conjugate to a variety of useful molecules and substances such as polyamines, polyamine acids, polypeptides, proteins, fluorescent dyes, intercalating dyes, reporter molecules, biotin, polysaccharides, monosaccharides, solid support materials, magnetic beads, dendrimer particles, DEAE-Sephadex<sup>TM</sup> (Pharmacia, Inc.), and the like. Depending on the specific compound of the invention and

the substance to be conjugated thereto, conjugation can occur using a compound of the invention as an alkylating agent, using a free amine thereof to react with an amine-reactive group of the substance to be conjugated, or by the use of cross-linking agents.

The present invention also provides compositions and methods for transfecting eukaryotic cells comprising a cationic lipid and an enveloped virus, a component of an enveloped virus, or a fusagenic peptide. Transfecting compositions comprise a cationic lipid compound of the invention in combination with an active or inactive enveloped virus, a viral component of an enveloped virus, or a non-viral fusagenic peptide that functions to facilitate entry of cationic lipid aggregates into the cell. Transfection compositions also optionally contain agents which inhibit lysosomal enzymes or enhance release of material from endosomes, such as chloroquine.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel cationic lipids having unique properties and advantages not heretofore available to the liposome art. The compounds can be used alone or in combination with other compounds, for example, DOPE, to prepare liposomes and other lipid aggregates suitable for transfection or delivery of compounds other than DNA to target cells, either in vitro or in vivo.

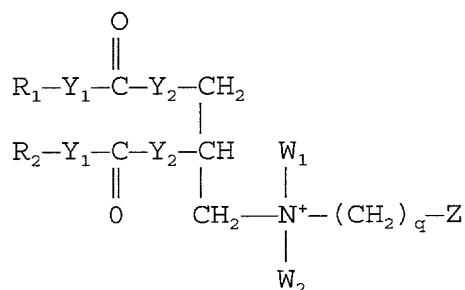
Compounds of the invention having a halogen substituent (Z is  $Z_2$  or  $Z_7$ ) are additionally useful for synthesis of more complex cationic lipids having the halogen replaced by a desired compound. The convenience of the halogen as a useful leaving group makes such substitutions straightforward. Examples of useful substituents include, without limitation, reporter groups, proteins, peptides, polypeptides, antibodies, carbohydrates, polysaccharides, and the like. Reporter groups can be any

readily analyzed or visualized molecule, including, without limitation, fluorescent tags (Fluorescein, rhodamine), luminescent tags (4-methoxy-4-(3-phosphatophenyl)-spiro[1,2-dioxetane-3,2'-adamantane] (PPD)) biotin, dyes, chelators, affinity probes, etc. Such reporters enable visualization and measurement of target cell-lipid aggregate interactions. Such reporters also provide a means for subsequently accessing targeted cells, by providing surface binding sites unique to targeted cells. In addition, certain drugs and therapeutic compounds can be substituted at the halogen site, by a metabolizable linkage, thereby enhancing efficiency of drug delivery. Also, DNA intercalating compounds and nucleic acid binding substances can be substituted, providing further DNA binding and enhancing transfection efficiency. Compounds such as lectins can be substituted, thereby enhancing the range of delivery capabilities.

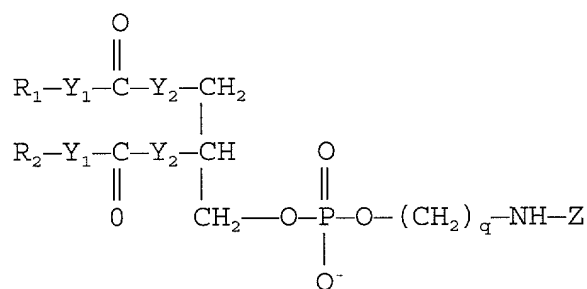
Compounds of the invention having ester-amide or amide linked lipophilic groups are particularly useful for applications demanding metabolizable, less toxic compounds. Most of the less toxic lipids currently available contain ester linker bonds, which can be metabolized and catabolized into other lipid species in the treated cells. However, cationic lipids containing ester linker bonds are not stable when stored in an aqueous solution. More stable compounds typically contain ether bonds which are not readily hydrolyzable in the cell. The present invention resolves the problems associated with prior art compounds by providing delivery agents which are stable in aqueous solution, but are metabolizable and thus less toxic to the target cell.

Of those compounds in which the lipophilic group is attached via ester-amide or amide linker bonds, this invention includes, but is not limited to, compounds represented by formulas II and III:

Formula II



Formula III

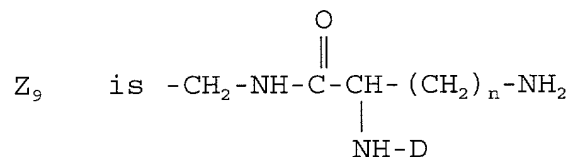
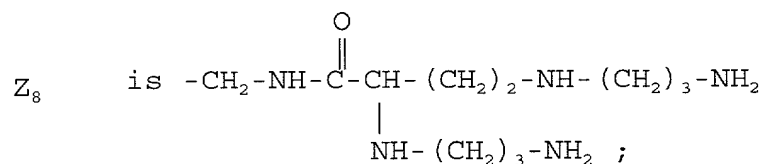


In formula II:

$\text{R}_1$  and  $\text{R}_2$ , independently of one another, are  $\text{C}_{1-23}$  alkyl or alkenyl;  $\text{Y}_1$  and  $\text{Y}_2$ , independently of one another, are O and N, wherein at least one of  $\text{Y}_1$  and  $\text{Y}_2$  is N; and  $\text{W}_1$  and  $\text{W}_2$ , independently of one another, are  $\text{C}_{1-24}$  branched or straight chain alkyl, alkenyl or aryl;  $q$  is 1 to 6; and  $\text{Z}$  is selected from the group consisting of  $\text{Z}_1\text{-Z}_{13}$  and  $\text{Z}_{18}$  where

- $\text{Z}_1$  is H;
- $\text{Z}_2$  is  $\text{-(CH}_2\text{)}_n\text{-X}$ , where  $n = 1\text{-}24$  and  $\text{X}$  is selected from the group consisting of Br, Cl, I and F;
- $\text{Z}_3$  is  $\text{-(CH}_2\text{)}_n\text{-NH}_2$ ,  $n = 1\text{-}24$ ;
- $\text{Z}_4$  is  $\text{-CH}_2\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_4\text{-NH}_2$ ;
- $\text{Z}_5$  is  $\text{-CH}_2\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_3\text{-NH}_2$ ;
- $\text{Z}_6$  is  $\text{-CH}_2\text{-NH-(CH}_2\text{)}_n\text{-NH}_2$ ,  $n = 2\text{-}24$ ;

Z<sub>7</sub> is -L-X where L is selected from the group consisting of branched or straight chain alkyl, alkenyl, cycloalkyl, aryl, alkoxy, thioalkyl and thioether groups having from 1 to about 24 carbon atoms, and X is selected from the group consisting of Br, Cl, I, F, NH<sub>2</sub> and [(NH<sub>2</sub>)-(CH<sub>2</sub>)<sub>n</sub>]<sub>m</sub> where n is 2-24 and m is 1-24;



where n = 1-24, D is H or other groups attached by amide or alkyl amino groups;

Z<sub>10</sub> is a reporter molecule;

Z<sub>11</sub> is a protein, peptide or polypeptide;



$Z_{12}$  is a polysaccharide;  
 $Z_{13}$  is an amine or halide reactive group; and  
 $Z_{18}$  is a nucleic acid binding substance.

Of particular interest are the products of Formula II in which  $W_1$  and  $W_2$  are methyl groups and  $q$  is 1.

In formula III:

$R_1$ ,  $R_2$ ,  $Y_1$  and  $Y_2$  are as defined above for Formula II,  $q$  is 2-6 with  $q$  of 2 being preferred; and  $Z$  is selected from the group consisting of  $Z_1$ ,  $Z_3$ ,  $Z_7$ ,  $Z_{10}$ - $Z_{12}$ ,  $Z_{14}$ - $Z_{15}$ , and  $Z_{18}$  where

$Z_1$  is H,  
 $Z_3$  is  $-(CH_2)_n-NH_2$ ,  $n = 1-24$ ,  
 $Z_7$  is  $-L-X$  where  $L$  is selected from the group consisting of branched or straight chain alkyl, alkenyl, cycloalkyl, aryl, alkoxy, thioalkyl and thioether groups having from 1 to about 24 carbon atoms, and  $X$  is selected from the group consisting of Br, Cl, I, F,  $NH_2$  and  $[(NH_2)-(CH_2)_n]_m$  where  $n$  is 2-24 and  $m$  is 1-24,  
 $Z_{10}$  is a reporter molecule,  
 $Z_{11}$  is a protein, peptide or polypeptide,  
 $Z_{12}$  is a polysaccharide,

$Z_{14}$  is
 
$$\begin{array}{c}
 O \\
 || \\
 -C-CH-(CH_2)_3-NH-(CH_2)_3-NH_2 \\
 | \\
 NH-(CH_2)_3-NH_2
 \end{array}$$

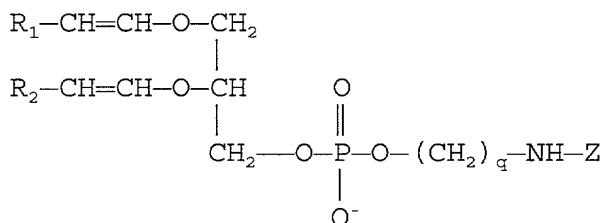
$Z_{15}$  is
 
$$\begin{array}{c}
 O \\
 || \\
 -C-CH-(CH_2)_n-NH_2 \\
 | \\
 NH-D
 \end{array}$$

$n = 1-24$ ,  $D$  is H or other groups attached by amide or alkyl amino groups, and

$Z_{18}$  is a nucleic acid binding substance.

Compounds of the invention having an enol-ether linked lipophilic group are particularly useful in pH-controlled delivery of macromolecules. The enol-ether compounds of the invention are susceptible to acid hydrolysis but generally stable towards bases. Of those compounds in which the lipophilic group is attached via enol-ether linker bonds, this invention includes, but is not limited to, compounds represented by Formula IV:

Formula IV



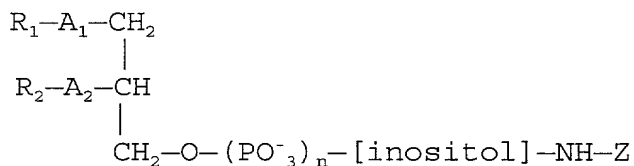
In Formula IV:

$R_1$  and  $R_2$ , independently of one another, are  $C_{1-23}$  alkyl or alkenyl;  $q = 2-6$  with  $q$  of 2 being preferred; and  $Z$  is selected from any of  $Z_1$ ,  $Z_3$ ,  $Z_7$ ,  $Z_{10}-Z_{12}$  and  $Z_{14}-Z_{15}$ , as defined above for Formula III.

Also included in this invention are compounds of formula I wherein the spacer arm comprises inositol or phosphoinositol. Compounds of the invention having an inositol spacer arm are particularly useful in effectively transfecting cells that contain receptors for inositol phosphates. Since phosphoinositides are found in both plants and animals, the cationic analogues have a broad delivery range. Compounds

comprising an inositol or phosphoinositol spacer arm include, but are not limited to, compounds represented by formula V:

Formula V

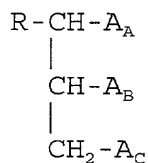


In Formula V:

$R_1$  and  $R_2$ , independently of one another, are  $C_{1-23}$  alkyl or alkenyl;  $A_1$  and  $A_2$ , independently of one another, are -O- or -CO<sub>2</sub>-;  $n$  is 1 or 2, with  $n$  of 1 being preferable; and  $Z$  is selected from any of  $Z_1$ ,  $Z_3$ ,  $Z_7$ ,  $Z_{10}$ - $Z_{12}$ ,  $Z_{14}$ - $Z_{15}$ , and  $Z_{18}$ , as defined above for Formula III.

Additional subsets of compounds of formula I of this invention include cationic ceramides and sphingolipids. These compounds differ from the cationic lipids currently in use by lacking the glycerol (1,2-propanediol) spacer arm and the linker bond attaching the lipophilic moiety to the spacer arm is an alkyl bond, rather than an easily hydrolyzable bond such as an ester or amide. Since ceramides and sphingolipids are present in nervous tissues, cationic analogues of these compounds are particularly useful for transfecting neural cells. The cationic ceramide and sphingolipid compounds of the invention include, but are not limited to, compounds represented by formula VI:

Formula VI



In Formula VI:

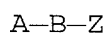
R is a straight-chain or branched alkyl or alkenyl having one to about 24 carbon atoms. Preferred R groups are 1-alkenyl having about 12 to about 22 carbon atoms, with 1-pentadecene being most preferred.

$A_A-A_C$ , independently of one another, are selected from the group consisting of  $A_1-A_4$ , wherein one of  $A_A-A_C$  is  $A_1$ , one of  $A_A-A_C$  is  $A_2$ , and one of  $A_A-A_C$  is  $A_3$  or  $A_4$ , and wherein the groups  $A_1-A_4$  are as follows:

- $A_1$  is OH;
- $A_2$  is -NH-R, where R is  $C_{1-23}$  alkyl, alkenyl or acyl;
- $A_3$  is -O-Z or -NH-Z, where Z is selected from the group consisting of  $Z_1-Z_{13}$  as defined above for Formula II; and
- $A_4$  is  $-PO_4^--(CH_2)_n-NH-Z$ , where n is 1-24, with n of 2 being preferred, Z is selected from any of  $Z_1, Z_3, Z_7, Z_{10}-Z_{12}, Z_{14}-Z_{15}$  and  $Z_{18}$ , as defined above for Formula III.

Also included in this invention are compounds of Formula I wherein the lipophilic group is a steroid, rather than a long-chain hydrocarbon. Preferred steroids include stigmasterol, ergosterol and cholic acid. These compounds are useful in transfecting diverse types of cells. Cationic steroids of the invention include, but are not limited to, compounds represented by formula VII:

Formula VII



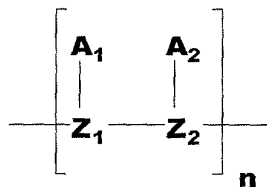
In Formula VII:

A is a steroid selected from the group consisting of stigmasterol, ergosterol or cholic acid; B is -NH-CH<sub>2</sub>- or -CO-N-R<sub>1</sub>, where B is -NH-CH<sub>2</sub>- when A is cholic acid and B is -CO-N-R<sub>1</sub>- when A is stigmasterol or ergosterol; where R<sub>1</sub> is an alkyl, alkenyl, alkynyl, alkoxy, acyl or alkythio having from 1 to about 24 carbon atoms; and where Z is selected from any of Z<sub>3</sub>-Z<sub>13</sub> and Z<sub>18</sub> as defined above for general Formula I.

The compounds of the present invention also include those in which the cationic region is an amino acid which is indirectly or directly attached, i.e. with or without a linker group, to the lipophilic moiety. These lipophilic polyamino acids are particularly useful for intracellular delivery of negatively charged macromolecules. This aspect of the invention is based on the premise that polycationic polyamino acids alkylated with long hydrocarbon chains have enhanced affinity for cells, many of which bear a net negative charge, and for various polyanions, such as nucleic acids, relative to normal polyamino acids. Moreover, because of the relatively high lipid content of the alkylated polyamino acids, these compounds interact more strongly with the lipid bilayer of cell membranes than their cognate polyamino acids.

Lipophilic polyamino acids of the invention include, but are not limited to, compounds represented by Formula VIII:

Formula VIII



In Formula VIII:

$Z_1$  and  $Z_2$ , independently of one another, are amino acids selected from the group consisting of ornithine, lysine, arginine and histidine;

$n$  is an integer (chain length) ranging from 1 to about 2,000 with  $n$  of between 10 and 50 being preferred.

$A_1$  and  $A_2$ , independently of one another, are selected from the group consisting of the following groups  $X_1$ - $X_6$ :

$X_1$  is a straight-chain alkyl, alkenyl, or alkynyl group having from 2 to about 22 carbon atoms wherein one or more non-neighboring  $-CH_2-$  groups can be replaced with an O or S atom;

$X_2$  is a branched alkyl, alkenyl, or alkynyl group having from 2 to about 22 carbon atoms wherein one or more non-neighboring  $-CH_2-$  groups can be replaced with an O or S atom;

$X_3$  is a straight-chain or branched alkyl group substituted with one or two OH, SH,  $NH_2$  or amine groups within about 3 carbon atoms of the bond between  $X_3$  and Z;

$X_4$  is a substituted straight-chain or branched alkyl, alkenyl or alkynyl group having from 2 to about 22 carbon atoms wherein the substituent is an aromatic, alicyclic, heterocyclic or polycyclic ring and wherein one or more of the non-neighboring  $-CH_2-$  groups of said alkyl, alkenyl or alkynyl group can be substituted with an O or S atom.

X<sub>5</sub> is a -B-L group wherein B is selected from the group -CO-, -CO<sub>2</sub>-, -OCO-, -CO-N-, -O-CO-N-, -O-CH<sub>2</sub>-, -CH<sub>2</sub>-O-, -S-CH<sub>2</sub>-, -CH<sub>2</sub>-S- or -CH<sub>2</sub>- and L is selected from the group consisting of

X<sub>1</sub>, X<sub>2</sub>, X<sub>4</sub>, or an aromatic, alicyclic, heterocyclic or polycyclic ring moiety;

X<sub>6</sub> is a -CH(D-L)<sub>2</sub> or a -C(D-L)<sub>3</sub> group wherein D is selected from the group consisting of -CO-, -CO<sub>2</sub>-, -OCO-, -CO-N-, -O-CO-N-, -O-, or -S- and L is selected from the group consisting of:

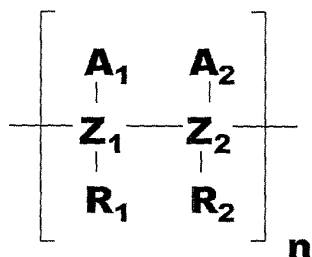
X<sub>1</sub>, X<sub>2</sub>, X<sub>4</sub>, or an aromatic, alicyclic, heterocyclic or polycyclic ring moiety.

Additional subsets of compounds of Formula I of this invention include those in which the polycationic region is a modified polysaccharide. The lipophilic polycationic polysaccharide compounds of the invention comprise a heteropolysaccharide or homopolysaccharide backbone, preferably a homopolysaccharide such as a glucan or galactan. The polysaccharide comprises between 2 and 2,000 monosaccharides, at least one of which has a cationic substituent. Preferred substituents include tertiary amines, most preferably diethylaminoethyl. Each monomeric unit has between about 3 and about 7 carbon atoms, preferably 3 to 6, and most preferably 6. Preferred polysaccharides include polymers of modified dextrans. Of particular interest are the products of general Formula I in which Z is a modified glucose, m is 0, n is 50 to 600, and R is C<sub>2-24</sub> alkyl or alkenyl. Most preferred is lipophilic DEAE-dextran. Lipophilic polycationic polysaccharides show increased affinity for negatively charged substances relative to the unsubstituted glycans. Moreover, because of the high lipid

content, the modified polysaccharides of the invention interact strongly with the lipid bilayer of cell membranes.

Lipophilic polycationic polysaccharides of the invention include, but are not limited to, compounds represented by Formula IX:

FORMULA IX



where  $Z_1$  and  $Z_2$ , independently of one another, are monosaccharides, preferably glucose;  $n$  is an integer (chain length) ranging in value from 1 to about 600, with  $n$  of between 50 and 200 being preferred;  $A_1$  and  $A_2$ , independently of one another, are tertiary amines, preferably diethylaminoethyl; and  $R_1$  and  $R_2$ , independently of one another, are selected from the group consisting of groups  $X_1$ - $X_6$ , as defined above for Formula VIII.

This invention also includes lipid aggregates comprising one or more of the compounds of Formulas II-IX or mixtures thereof. Of particular interest are lipid aggregates of the compounds of Formulas II-IX which most closely simulate their naturally occurring counterparts.

The transfection methods of the present invention employing compounds of formulas II-IX or mixtures thereof can be applied to in vitro and in vivo transfection of cells, particularly to transfection of eukaryotic cells including animal cells. The methods of this invention can be used to generate transfected



cells which express useful gene products. The methods of this invention can also be employed as a step in the production of transgenic animals. The methods of this invention are useful as a step in any therapeutic method requiring the introduction of nucleic acids into cells. In particular, these methods are useful in cancer treatment, in in vivo and ex vivo gene therapy, and in diagnostic methods. The transfection compositions of this invention can be employed as research reagents in any transfection of cells done for research purposes. Nucleic acids that can be transfected by the methods of this invention include DNA and RNA from any source comprising natural bases or non-natural bases, and include those encoding and capable of expressing therapeutic or otherwise useful proteins in cells, those which inhibit undesired expression of nucleic acids in cells, those which inhibit undesired enzymatic activity or activate desired enzymes, those which catalyze reactions (Ribozymes), and those which function in diagnostic assays.

The compositions and methods provided herein can also be readily adapted in view of the disclosure herein to introduce biologically active anionic macromolecules other than nucleic acids including, among others, polyamines, polyamine acids, polypeptides, proteins, biotin, and polysaccharides into cells. Other materials useful, for example as therapeutic agents, diagnostic materials and research reagents, can be complexed by the cationic lipid aggregates and introduced into cells by the methods of this invention.

The present invention also provides compositions and methods for transfecting cells, preferably eukaryotic cells, comprising a cationic lipid and an enveloped virus, a component of an enveloped virus, a membrane virus, or a non-viral fusagenic compound. These transfecting compositions comprise a cationic lipid compound of the invention in combination with an active or inactive enveloped virus, a viral component of an enveloped

virus, or a non-viral fusagenic peptide that functions to facilitate entry of cationic lipid aggregates into the cell.

5 Viral components of enveloped viruses useful in transfection compositions include viral proteins, envelope fusion peptides, particular viral spike glycoproteins, multimers (i.e., dimers and trimers) thereof, viral peptides of viral spike glycoproteins, and viral envelope fragments containing embedded viral protein. The use of viral components for enhanced transfection is exemplified with an inactive Semliki Forest virus (SFV) particle in co-pending U.S. patent application 08/090,290 (filed July 12, 1993), which is incorporated herein by reference in its entirety. Viruses with a broad host range, such as alphaviruses, are generally preferred. However, viruses which exhibit cell specificity or a specific host range are particularly useful for targeted delivery.

15 The mechanism of viral enhancement of cationic lipid/DNA complex delivery is not fully understood. After the initial viral recognition of the host cell, certain viruses enter the cell by direct fusion with the cell membrane and, in certain cases, after neuraminidase cleavage of polysaccharides on the cell surface (influenza virus). The viral nucleic acid is then released into the cytoplasm. Other viruses enter the cell through endocytosis and are subsequently released from the endosomal compartment. All steps are protein mediated. Although the mechanism is not understood, these proteins, in conjunction with the cationic lipid/DNA complexes, enhance the uptake of macromolecules by the cell.

25 Inclusion of an enveloped virus, such as SFV, in a transfection composition with cationic lipid aggregates complexed with nucleic acids thus may enhance transfection compared to transfection mediated by the cationic lipid alone. Enhancement of transfection by alphaviruses, particularly SFV, is pronounced

in historically hard-to-transfect cell lines, including human primary cell lines. Enhancement of transfection by enveloped viruses occurs in any cell which the virus can enter and infect. Enhancement of transfection of alphaviruses, particularly SFV, occurs in cells which comprise cholesterol or another 3  $\beta$ -OH-sterol in their cell membrane. Methods for introducing a sterol or increasing the level of a sterol in cell membranes are known in the art and described in the above-cited patent application (USSN 08/090,290).

Non-viral fusagenic peptides (or membrane fusion proteins) have been implicated in cell fusion reactions. Although the mechanism is not fully understood, fusagenic peptides can be added to transfection compositions comprising cationic compounds of the present invention to enhance the efficiency of transfection.

The cationic lipids, viral or non-viral enhancing agents and nucleic acid of transfecting compositions can be combined in a variety of ways prior to contact with cells, as described, for example, in the cited copending U.S. application. Transfection compositions also optionally contain agents which inhibit lysosomal enzymes or enhance release of material from endosomes, such as chloroquine.

This invention also includes transfection kits which include one or more of the compounds of formulas II-IX or mixtures thereof as cationic lipids. The invention also includes transfection kits comprising one or more of the compounds of formulas II-IX (or mixtures thereof) in combination with a viral agent, a component of an enveloped virus, or a non-viral fusagenic peptide.

## Definitions

Lipid Aggregate is a generic term which includes liposomes of all types both unilamellar and multilamellar as well as micelles and more amorphous aggregates of cationic lipid or lipid mixed with amphiphatic lipids such as phospholipids and steroids.

Target Cell refers to any cell to which a desired compound is delivered, using a lipid aggregate as carrier for the desired compound.

Transfection is used herein to mean the delivery of expressible nucleic acid to a target cell, such that the target cell is rendered capable of expressing said nucleic acid. It will be understood that the term "nucleic acid" includes both DNA and RNA without regard to molecular weight, and the term "expression" means any manifestation of the functional presence of the nucleic acid within the cell including, without limitation, both transient expression and stable expression.

Delivery is used to denote a process by which a desired compound is transferred to a target cell such that the desired compound is ultimately located inside the target cell or in, or on, the target cell membrane. In many uses of the compounds of the invention, the desired compound is not readily taken up by the target cell and delivery via lipid aggregates is a means for getting the desired compound into the cell. In certain uses, especially under in vivo conditions, delivery to a specific target cell type is preferable and can be facilitated by compounds of the invention.

Transfection-enhancing agent as used herein refers to any substance which, when used in conjunction with a cationic lipid, provides significant enhancement of transfection (2-fold or more) over transfection compositions comprising the cationic lipid alone.

Inactive virus refers to a virus which, after exposure to certain chemical or physical conditions, is no longer capable of expressing its viral RNA. Viral inactivation is assessed by exposing viral particles whose RNA contains a reporter gene under the control of the viral subgenomic promoter to potentially inactivating conditions.

Non-viral fusagenic peptides (or membrane fusion proteins) refer to proteins or protein assemblies which interact with lipid components of two opposed bilayers of cell membranes so as to bring about their unification. Although the mechanism is not fully understood, the following non-viral fusagenic compounds have been implicated in cell fusion reactions: melitrin (the principal toxic peptide in bee venom), GALA (a synthetic peptide), snake venom cardiotoxins, snake venom curaremimetic neurotoxins, myelin basic protein, bindin and lysin (proteins from abalone spermatozoa), prostaglandins, FUS1 and FUS2 (products of the FUS1 and FUS2 genes of Saccharomyces cerevisiae), PH-30 (a complex of two proteins involved in sperm/egg fusion), members of the cadherin family, and variants of the neural cell adhesion molecule (NCAM). Compounds involved in cell fusion reactions may be added to compositions comprising compounds of the invention, thereby enhancing the efficiency of transfection.

The cationic lipids were prepared by following the general reaction schemes given below (Schemes 1-11).

The quarternized ammonium/carbamate lipids are synthesized as shown in Scheme 1. Alkyl isocyanate treatment of 3-dialkylamino-1,2-propanediol results in the formation of compound 1. Compound 1 is then alkylated with methyl chloride or methyl iodide to obtain compound I (Formula II,  $Z = Z_1$ ). Treatment of compound 1 with dihaloalkane results in compound II. Alkylation of compound 1 with bromoethylphthalamide followed by

hydrazinolysis results in compound III (Formula II,  $Z = Z_3$ ). Compounds IV-VI are synthesized by treatment of compound II with the corresponding amine at high temperature. Compounds IX and X are synthesized by condensing compound III with the corresponding amine-protected amino acids and the subsequent removal of the protecting group. Compound XI is synthesized by treating compound III with reporter groups that contain an activated carboxyl group.

The scheme provides a general method for the conjugation of lipids to any molecule or substance of interest. The alkyl halide II can be used as a general alkylating agent. Thus, any molecule of interest that has a nucleophilic moiety can react with compound II (Scheme 2) (J. March (1985) Advanced Organic Chemistry, John Wiley & Sons, New York, pp. 364-366; Hilgetag & A. Martini, eds. (1972) Preparative Organic Chemistry, John Wiley & Sons, New York, pp. 448-460). Macromolecules that contain amino groups such as proteins and antibodies can be conjugated to lipids in this manner. Smaller molecules that contain amino groups such as intercalators (methidium spermine), fluorescent dyes, nucleotides, nucleosides, amino acids, peptides and other reporter molecules such as biotin can also be conjugated in this manner.

Conversely, compounds III, IX-X, or IV-VI can be used for the conjugation of any molecules of interest that have electrophilic or nucleophilic sites. Compounds III, IX-X, or IV-VI can react with reporter molecules or other desired molecules if these molecules contain carboxylic acid sites, NHS ester or other active groups such as isothiocyanates, alkylhalides or chlorotriazines (Scheme 3) (Keezer, F. and Douraghi-Zdeh, K. (1967) Chem. Rev. 67:107; Dottario-Martin, B. and Ravel, J.H. (1978) Anal. Biochem. 76:562; Staros, J.V. (1982) Biochemistry 21:3950.

Compounds III, IX-X, or IV-VI can also be conjugated with molecules that contain nucleophilic sites such as amines by using cross-linking agents (Scheme 4). Disuccinimidyl suberate can be used to conjugate compounds III, IX-X, or IV-VI to molecules that contain an amino group (Staros, J.V. (1982) Biochemistry 21:3990). Cross-linking agents that contain NHS ester and maleimide can be used to conjugate compounds III, IX-X, or IV-VI to molecules that contain sulfhydryl group (Scheme 4) (Ji, T.H. (1979) Biochem. Biophys. Acta 559:39).

Phosphatidic carbamates (Formula III) are synthesized as shown in Scheme 5. Compound 1 is treated with alkyl isocyanate to give compound 2. Removal of the BOC protecting group from compound 2 gives compound XV. Treatment of compound XV with bromoethylphthalamide followed with hydrazinolysis results in XVI. Compound XVII is obtained by treating XVI with dihaloalkanes. Compounds XVIII and XIX are obtained by coupling XVI with BOC-protected carboxy spermine or other amino acids, followed with the removal of the protecting group. Compound XX is obtained by coupling XVI with proteins or peptides using cross linking agents.

Cationic analogs of sphingolipids wherein  $A_A-A_C$  are selected from the group consisting of  $A_1-A_3$  (Formula VI) can be synthesized as described in Scheme 6. Sphingosine (1) is alkylated to give compound 2. The primary hydroxyl group is selectively protected with dimethoxytrityl (DMTR) to obtain compound 3. Compound 3 is alkylated with bromoacetic acid in the presence of base to obtain compound 4. Treatment of compound 4 with diamino alkane in the presence of DCC results in compound 5.

Compound 3 can be acylated with acetyl chloride to obtain compound 9 which, on treatment with acid, yields compound 10.

Compound 10 is alkylated with bromoacetic acid followed with treatment with DCC/diamino alkane to obtain compound 12.

Compounds 5 and 12 can be acylated or alkylated as in Schemes 1-5 to obtain analogous compounds. Thus, treatment of compound 5 with BOC-protected carboxy spermine followed with treatment with acid results in the polycationic compound 6. Proteins or polypeptides can be conjugated to compounds 5, 8 or 12 using cross linking agents.

Cationic analogs of sphingolipids wherein one of  $A_A-A_C$  is  $A_4$  (Formula VI) can be synthesized as described in Scheme 7. Compound 1 is selectively acylated using N-hydroxysuccinimide (NHS) esters of fatty acids to obtain compound 2. Selective protection of the hydroxyl groups using dimethoxytritylchloride (DMTrCl) yields compound 3. DCC-mediated coupling of compound 3 with amine-protected aminoalkyl phosphate gives compound 4. Removal of the protecting groups yields compound 5. Compound 5 can be converted to the various analogues using the methods described above in Schemes 3 and 4.

The cationic steroid compounds of the invention are synthesized as shown in Scheme 8. For example, cholic acid is treated with a diaminoalkane in the presence of DCC to obtain compound 2. Compound 2 is then treated with BOC-protected carboxyspermine to give compound 3. Alternatively, compound 2 is conjugated to macromolecules such as proteins, polypeptides, polyamines, and polycationic compounds (see, e.g., group  $R_3$  in Scheme 2) by using a cross linking agent. Thus, compound 2 can be treated with cross linkers such as DSS followed with proteins or polypeptides, for example, to obtain compound 4.

Compounds of the invention in which the lipophilic group is attached via an enol-ether linker bond, such as compounds represented by formula IV, are prepared as shown in Scheme 9.



Treatment of compound 2 with an aldehyde (1) in the presence of acid followed with dehydration results in compound 3. The ester bond of compound 3 is hydrolyzed with base to obtain the corresponding alcohol 4. Compound 4 is then treated with compound 1 in the presence of acid to obtain compound 5. Amine-protected aminoalcohols (6) are conjugated to compound 5 using DCC as coupling agent to obtain compound 7. Removal of the protecting group from compound 7 yields compound 8. Compound 8 can be further modified as described above for schemes 1-5.

The phosphoinositide lipids (Formula V) are synthesized as shown in Scheme 10. The hydroxyl groups of inositol are protected using standard techniques (see, e.g., J. Chem. Soc. (1987) 423-429) to produce compound 1. Compound 1 is then coupled to compound 2 using DCC as an activator to obtain compound 3. The isopropylidene group of compound 3 is removed with a mild acid to obtain compound 4. Compound 4 is then treated with bromopropylphthalamide to obtain compound 5. Hydrazinolysis of compound 5 results in compound 6. Compound 6 can be further modified, as described above for schemes 1-5, to produce the various compounds of formula V. The phosphoinositide lipids thus can be conjugated to any molecule or substance of interest, as exemplified with the carbamate lipids.

Lipophilic polyamino acids of the invention (Formula VIII) are synthesized as shown, for example, in Scheme 11. Polylysine is partially acylated using compound 2 in the presence of a coupling agent (such as DCC) to produce the corresponding lipophilic polyamino acid. As will be appreciated by those skilled in the art, compound 2 can be any lipophilic moiety comprising an amine-reactive group. Alternatively, the polyamino acid can be modified as described above for Schemes 1-5.

Lipophilic polycationic polysaccharides of the invention (Formula IX) can be similarly synthesized using standard coupling

techniques as shown, for example, in Scheme 12. DEAE-dextran is partially acylated with R-CO-OH-DCC, where R is alkyl, alkenyl C<sub>12-24</sub>, and treated with compound 2 in the presence of DCC to obtain compound 3.

5

The compounds of the invention can be used in the same manner as are prior art compounds such as DOTMA, DOTAP, DOGS and the like. Methods for incorporating such cationic lipids into lipid aggregates are well-known in the art. Representative methods are disclosed by Felgner et al., supra; Eppstein et al. supra; Behr et al. supra; Bangham, A. et al. (1965) M. Mol. Biol. 23:238-252; Olson, F. et al. (1979) Biochim. Biophys. Acta 557:9-23; Szoka, F. et al. (1978) Proc. Natl. Acad. Sci. USA 75:4194-4198; Mayhew, E. et al. (1984) Biochim. Biophys. Acta 775:169-175; Kim, S. et al. (1983) Biochim. Biophys. Acta 728:339-348; and Fukunaga, M. et al. (1984) Endocrinol. 115:757-761. Techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion as perhaps the most commonly used. See, e.g., Mayer, L. et al. (1986) Biochim. Biophys. Acta 858:161-168. Microfluidization is used when consistently small (50 - 200 nm) and relatively uniform aggregates are desired (Mayhew, E., supra). Aggregates ranging from about 50 nm to about 200 nm diameter are preferred; however, both larger and smaller sized aggregates are functional.

Methods of transfection and delivery of other compounds are well-known in the art. The compounds of the present invention yield lipid aggregates that can be used in the same processes as those prior art compounds.

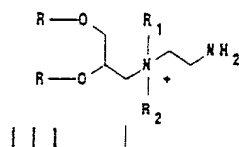
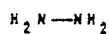
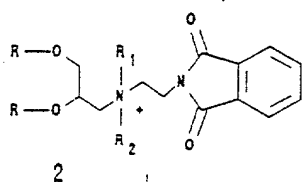
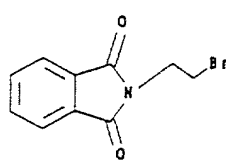
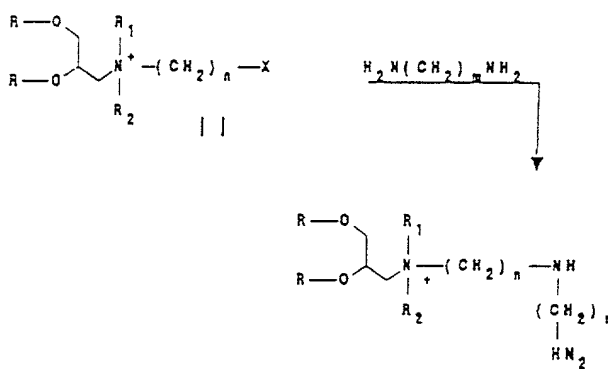
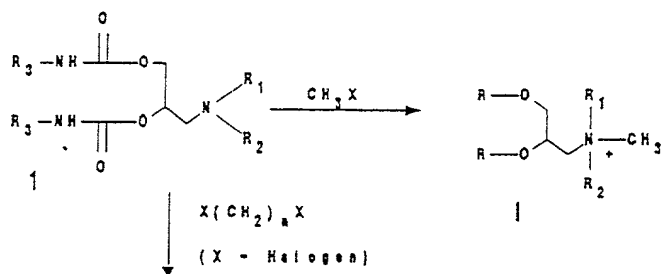
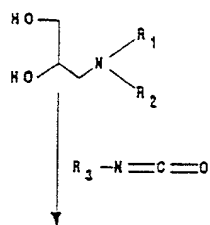
Viral components or non-viral fusagenic compounds can be combined with the cationic lipids of the invention to enhance the efficiency of transfection and/or the range of delivery capabilities. Methods for enhancing transfection using fusagenic

compounds are well-known in the art. Preferred methods include those disclosed in co-pending U.S. patent application 08/090,290.

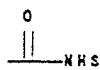
5 It will be readily apparent to those of ordinary skill in the art that a number of general parameters are important for optimal efficiency of transfection or delivery. These parameters include, for example, the cationic lipid concentration, the concentration of compound to be delivered, the medium employed for delivery, the length of time the cells are incubated with the  
10 polyanion-lipid complex, and the relative amounts of cationic and non-cationic lipid. It may be necessary to optimize these parameters for each particular cell type. Such optimization is routine employing the guidance provided herein and knowledge generally available to the art.

15 It will also be apparent to those of ordinary skill in the art that alternative methods, reagents, procedures and techniques other than those specifically detailed herein can be employed or readily adapted to produce the liposomal precursors and  
20 transfection compositions of this invention. Such alternative methods, reagents, procedures and techniques are within the spirit and scope of this invention.

# SCHEME 1



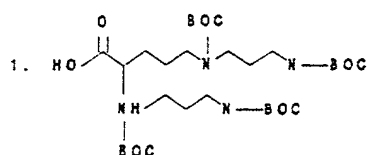
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DSS, Protein

(peptide)

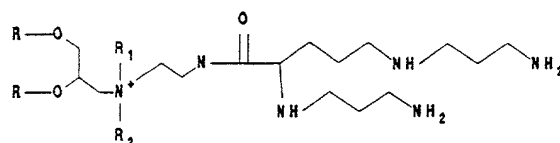
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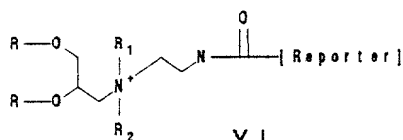
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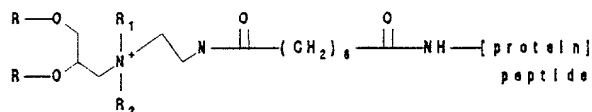
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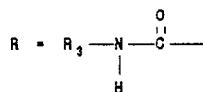
IX - X



XI



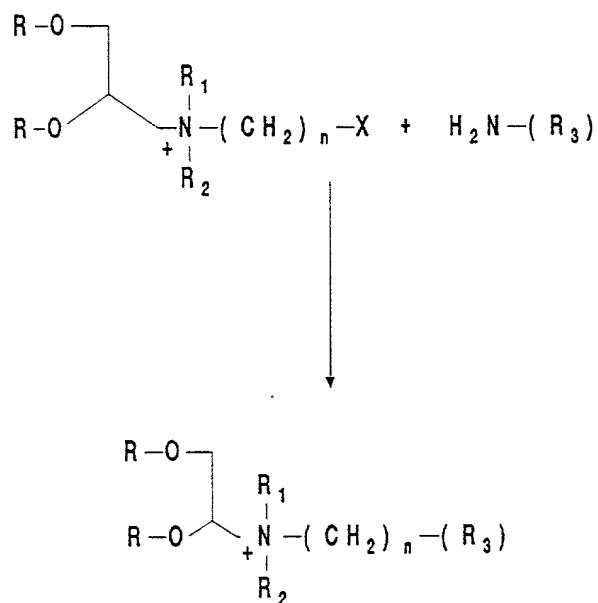
XII



R<sub>1</sub> and R<sub>2</sub> = C<sub>1-24</sub> alkyl, alkenyl or aryl

R<sub>3</sub> = C<sub>1-23</sub> alkyl or alkenyl

SCHEME 2



R = C<sub>1-24</sub> alkyl or alkenyl

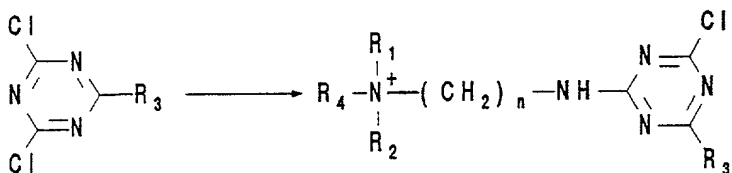
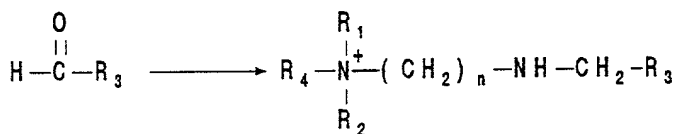
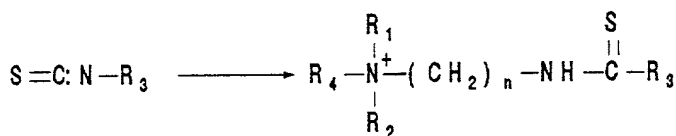
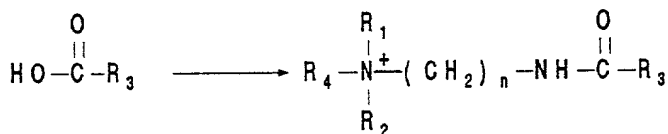
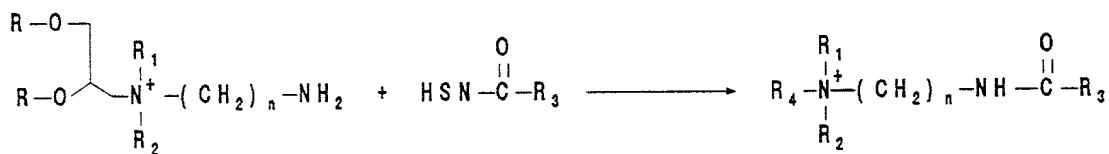
R<sub>1</sub> and R<sub>2</sub> = C<sub>1-24</sub> alkyl, alkenyl or aryl

R<sub>3</sub> = Polyamine  
 Reporter molecule  
 Protein  
 Polysaccharide  
 Polypeptide  
 Fluorescent dyes  
 Intercalators  
 Polyamino acid

n = 1-24

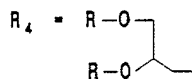
X = Halogen

SCHEME 3



R = C<sub>1-24</sub> alkyl  
or alkenyl

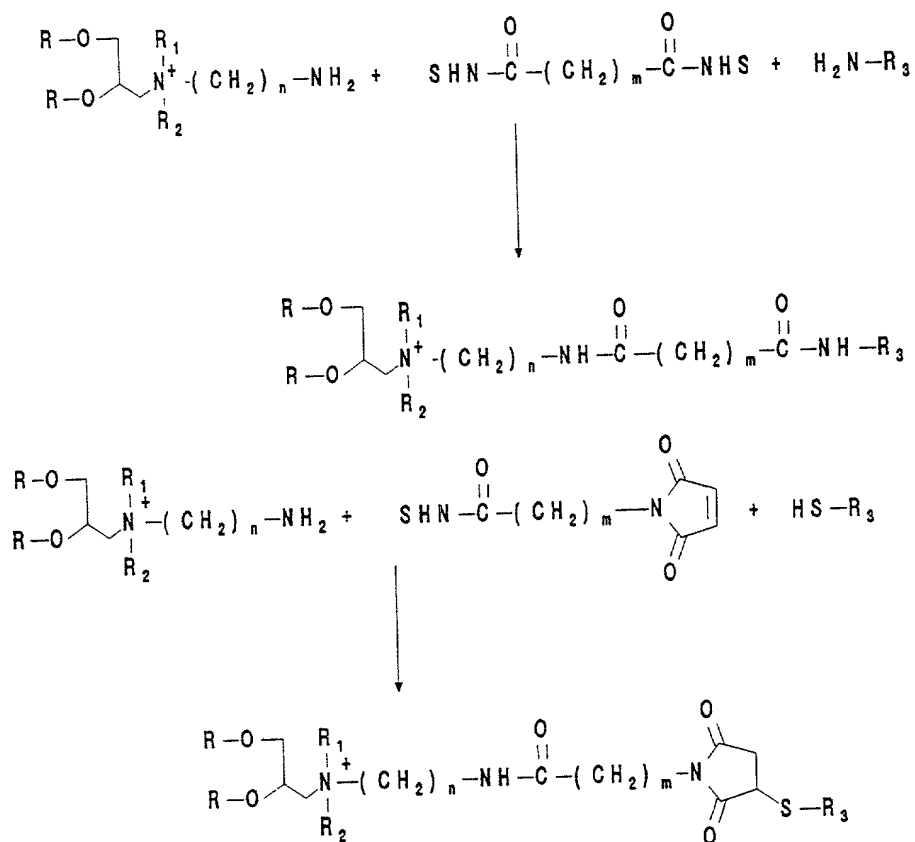
R<sub>1</sub> and R<sub>2</sub> = C<sub>1-24</sub> alkyl,  
alkenyl or  
aryl



R<sub>3</sub> = Polyamine  
Reporter molecule  
Protein  
Polysaccharide  
Polypeptide  
Fluorescent dyes  
Intercalators  
Polyamino acid  
Solid support  
Magnetic beads

n = 2-24

SCHEME 4



R = C<sub>1-24</sub> alkyl or alkenyl

R<sub>1</sub> and R<sub>2</sub> = C<sub>1-24</sub> alkyl, alkenyl or aryl

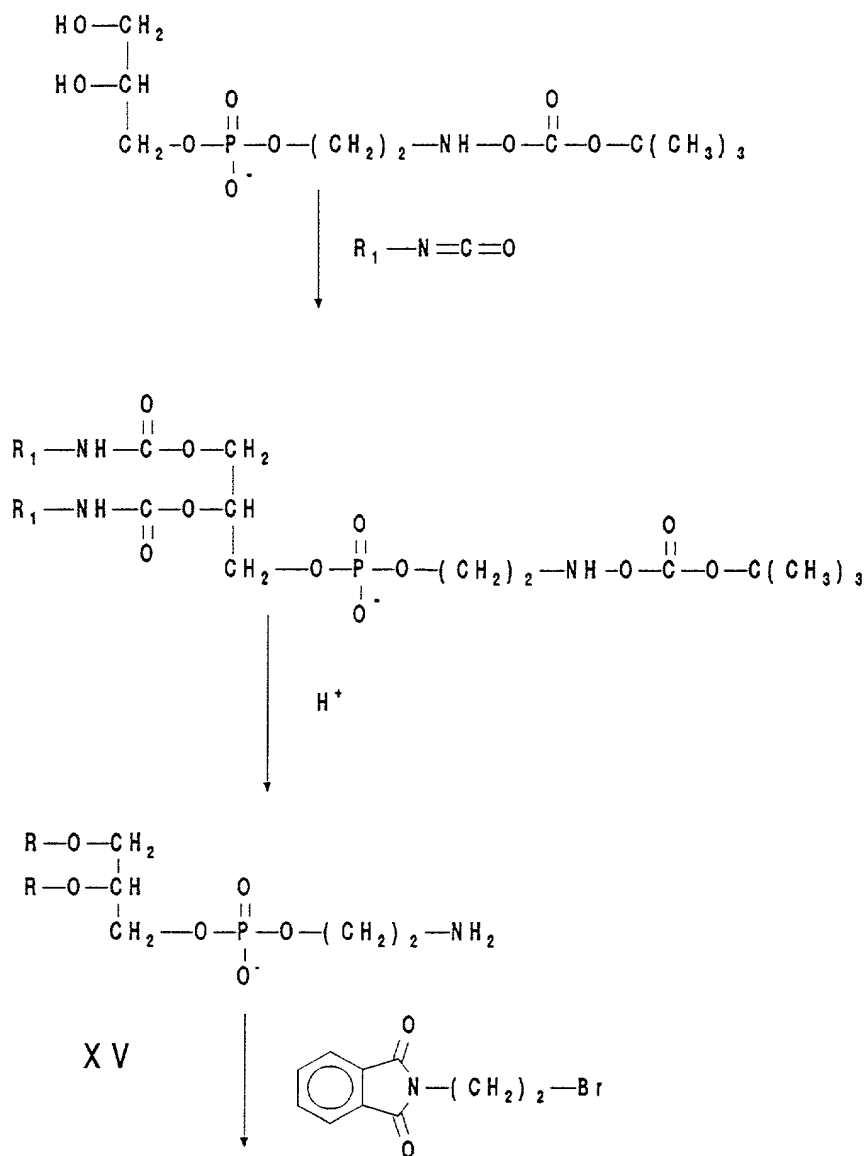
n = 2-24

m = 1-24

R<sub>3</sub> = Polyamine  
Reporter molecule  
Protein  
Polysaccharide  
Polypeptide  
Fluorescent dyes  
Intercalators  
Polyamino acid

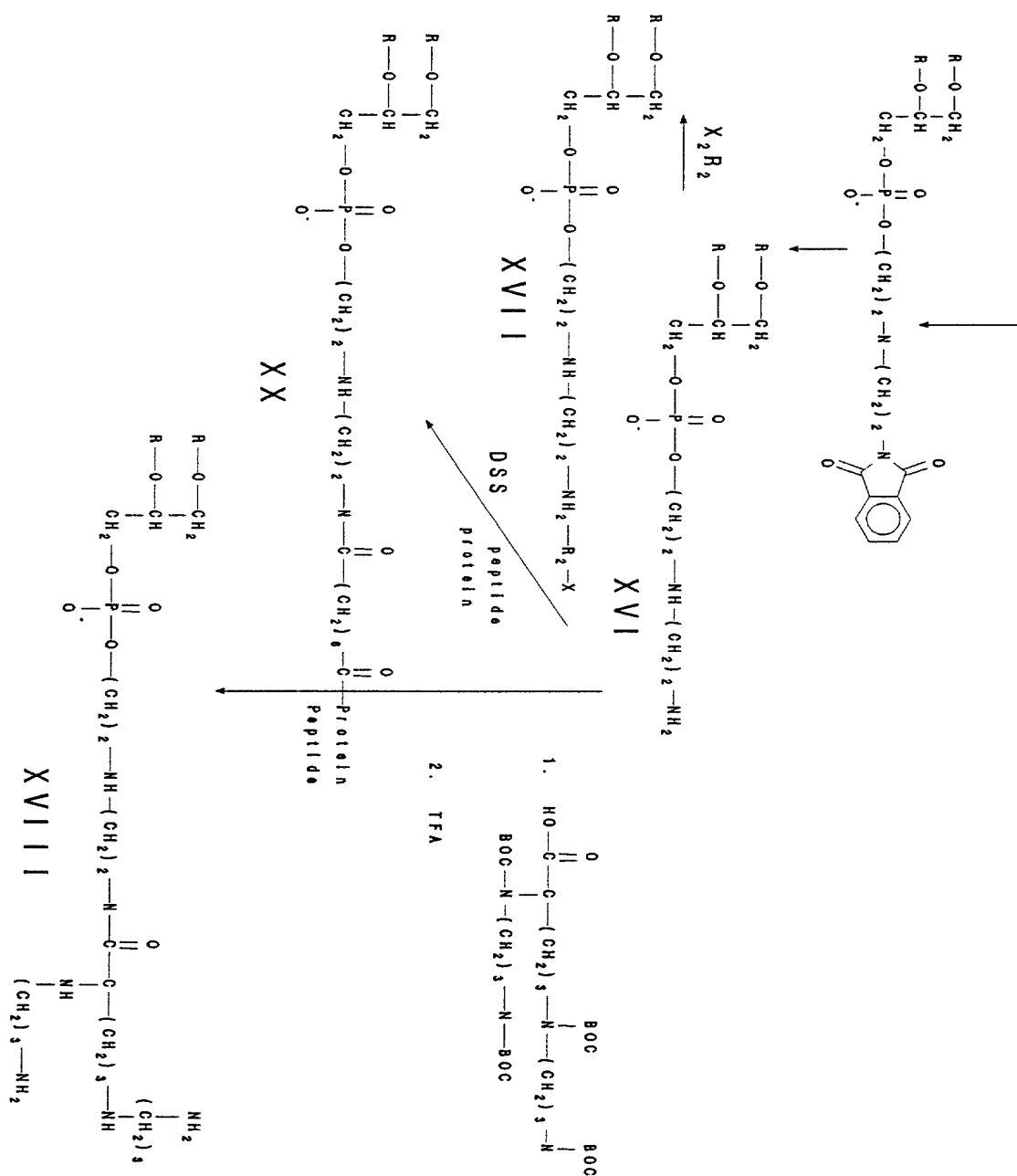


SCHEME 5

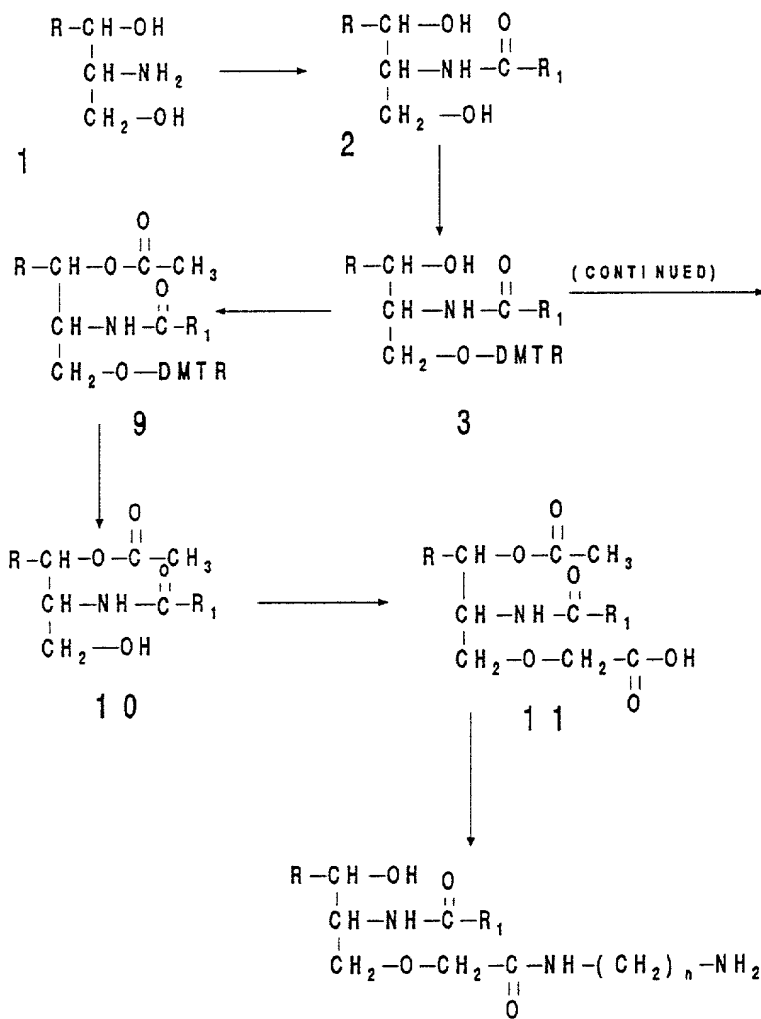


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## 40

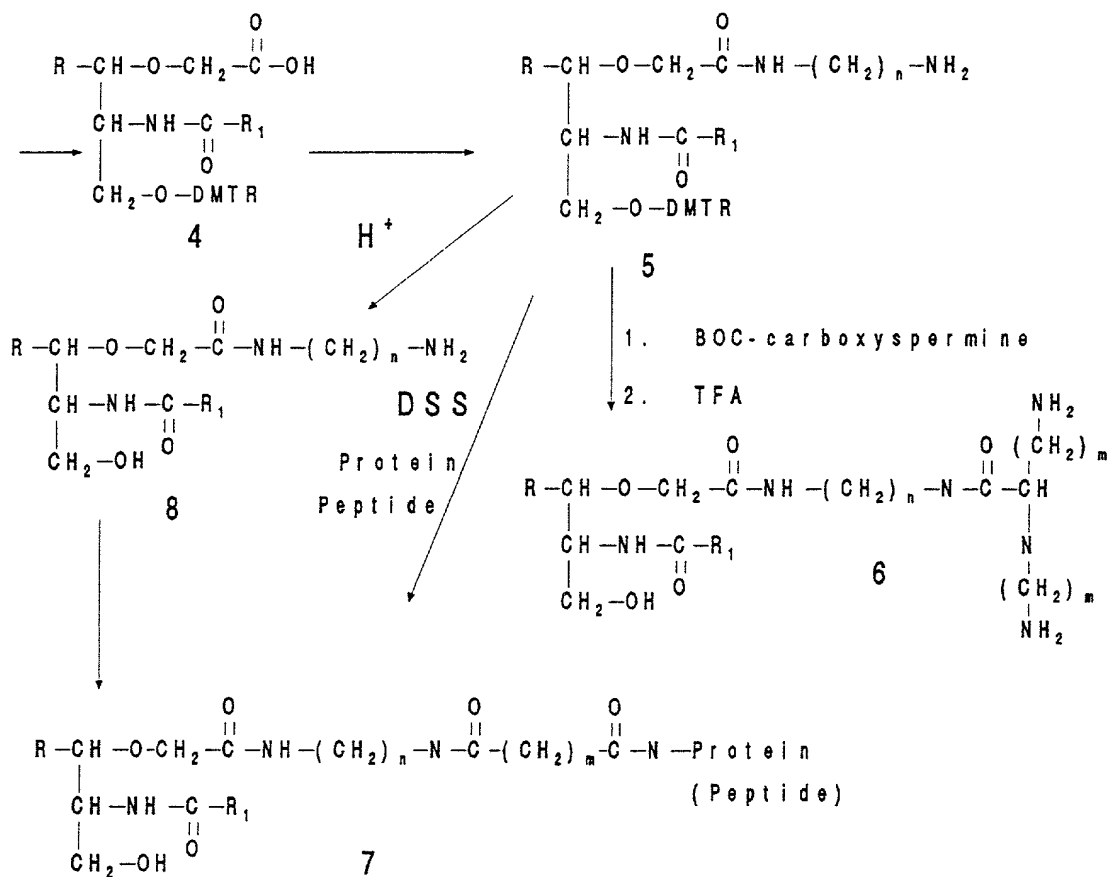


# SCHEME 6

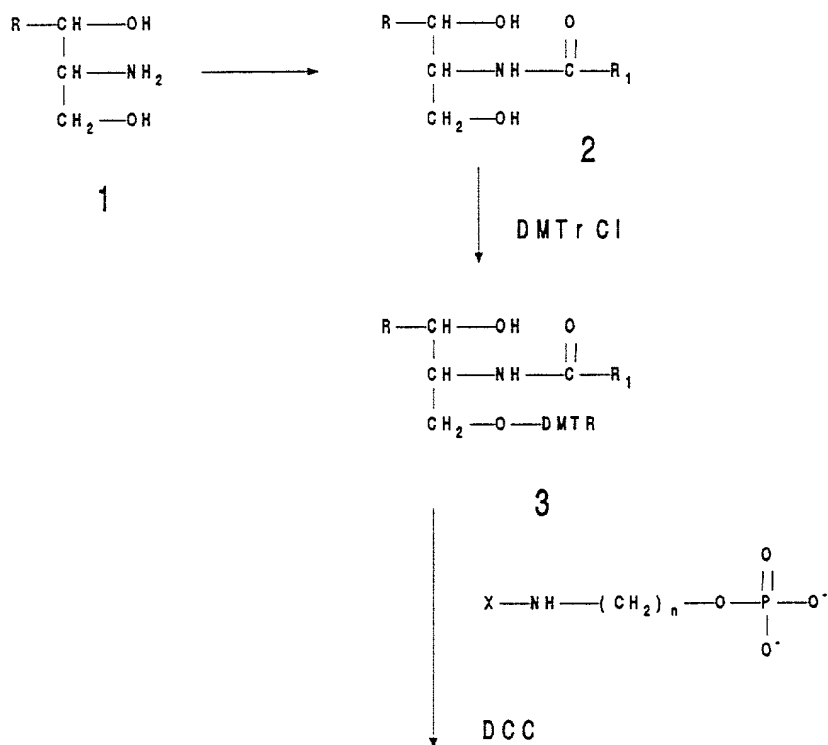


$\text{R} = \text{C}_{1-24}$  alkyl  
           or alkenyl  
 $\text{R}_1 = \text{C}_{1-23}$  alkyl  
           or alkenyl  
 $n = 2-24$   
 $m = 1-24$

SCHEME 6 (continued)



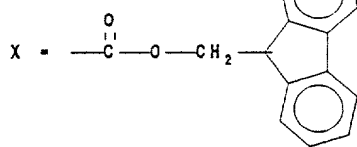
SCHEME 7



R = C<sub>1-24</sub> alkyl or alkenyl

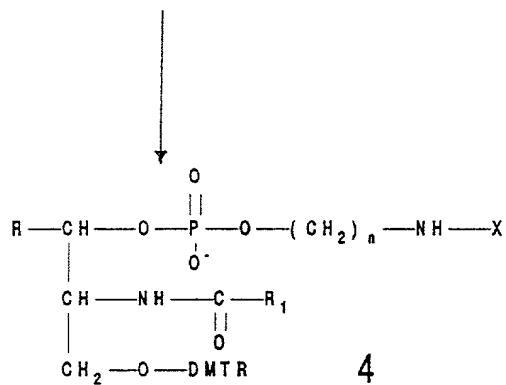
R<sub>1</sub> = C<sub>1-23</sub> alkyl or alkenyl

n = 1-24

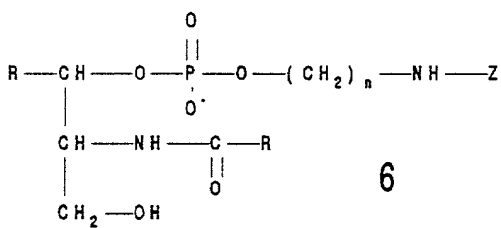
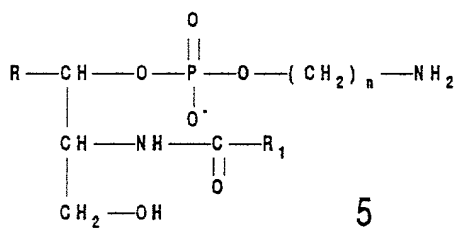


(continued)

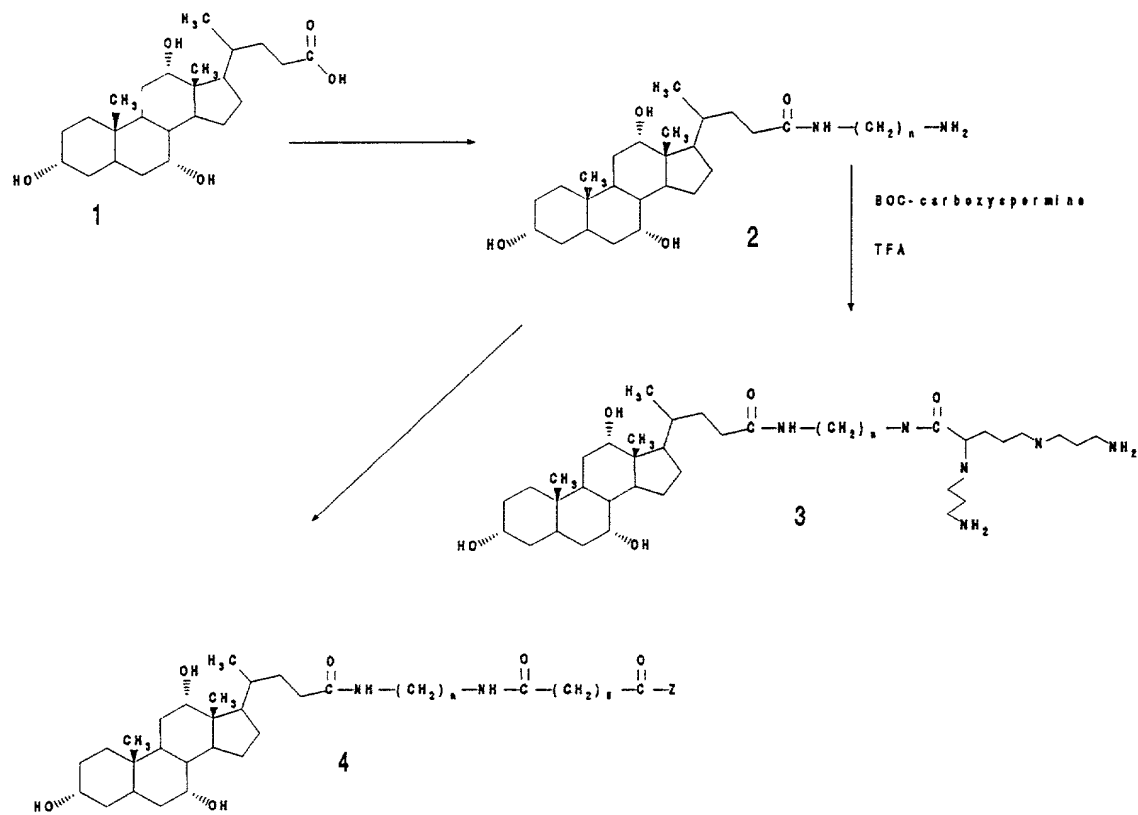
SCHEME 7 (Continued)



1.  $\text{OH}^-$
2.  $\text{H}^+$



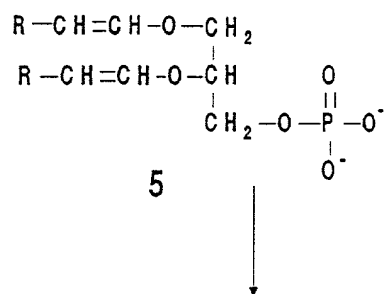
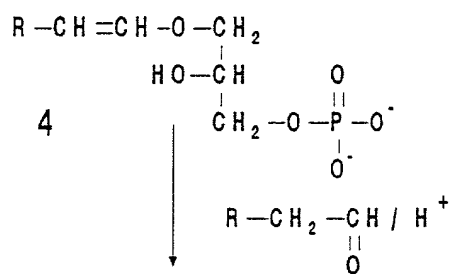
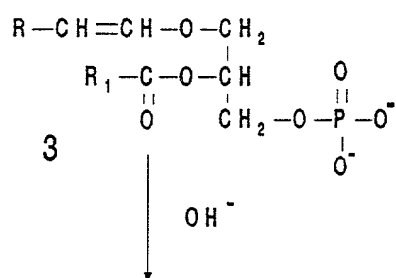
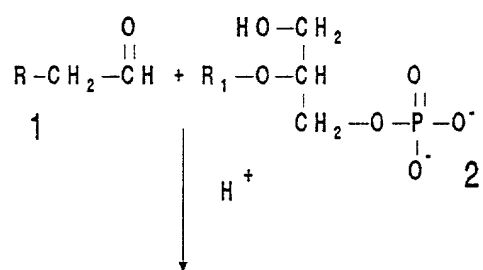
# SCHEME 8



$n = 2-24$

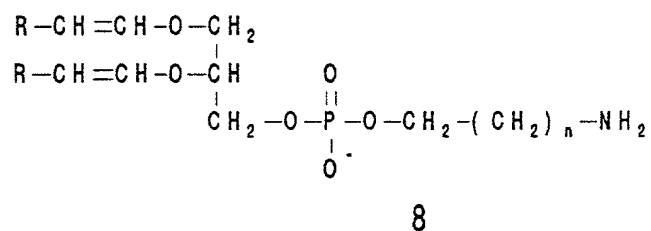
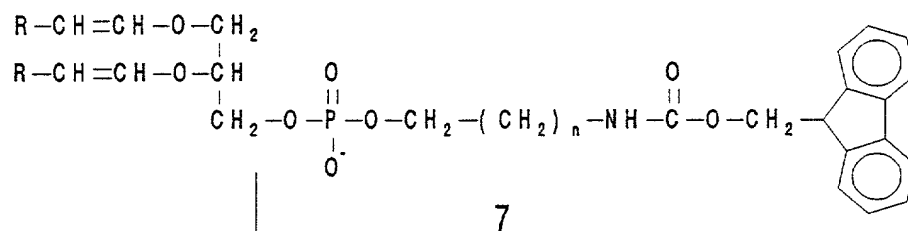
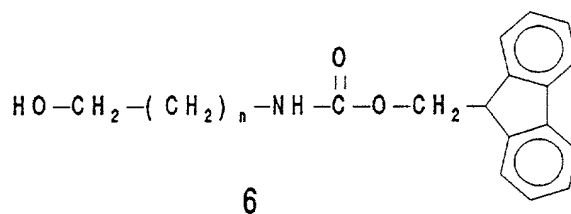
Z = protein, peptide, polypeptide or polycation

SCHEME 9

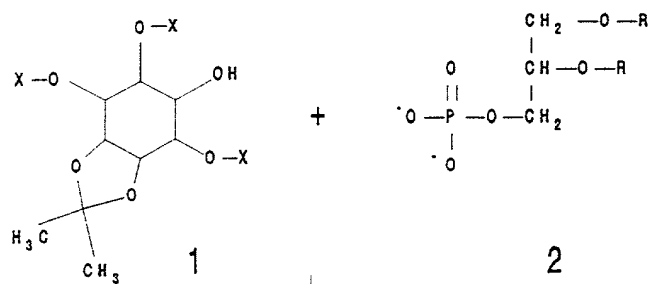




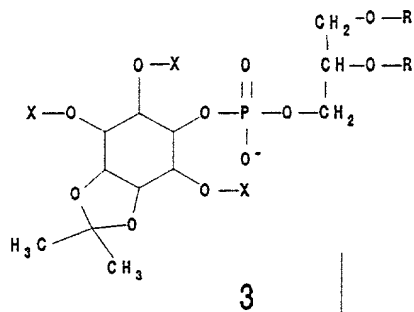
SCHEME 9 (continued)



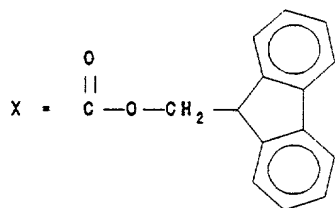
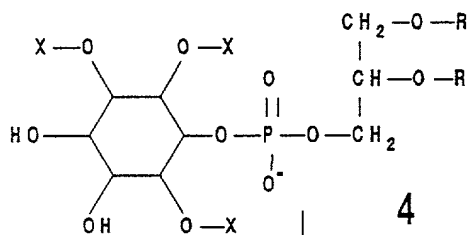
SCHEME 10



DCC

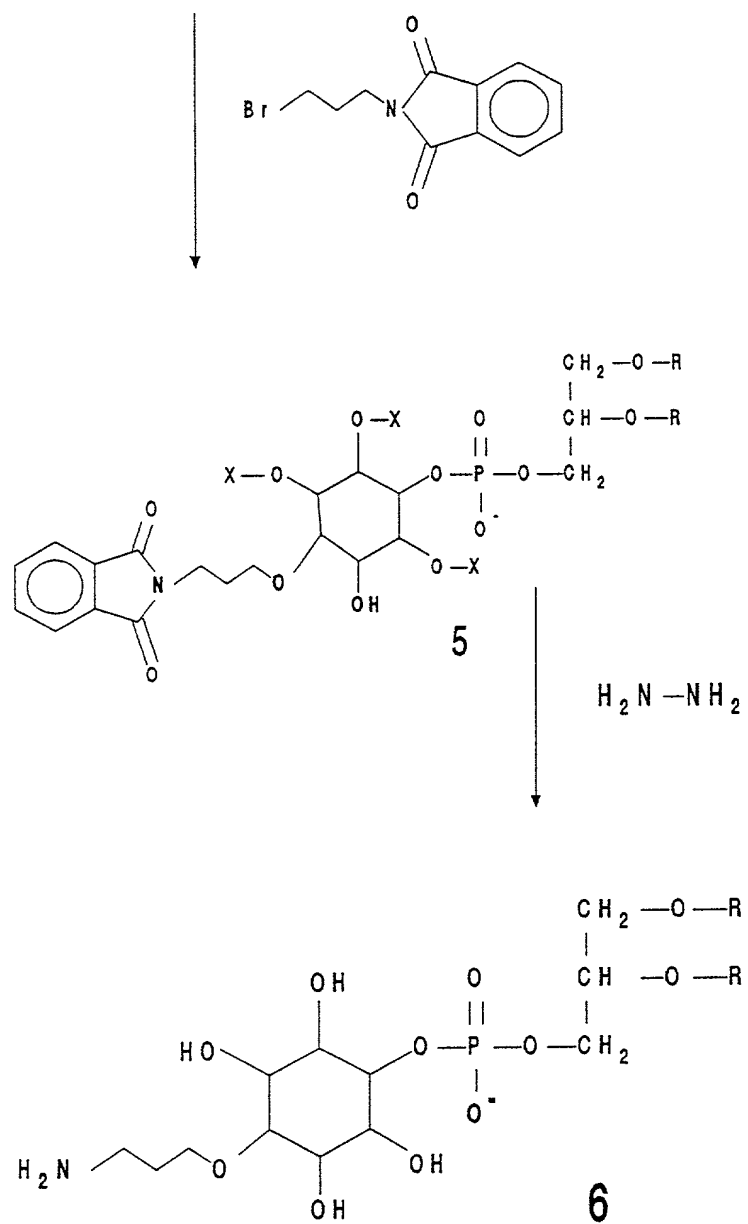


H<sup>+</sup>

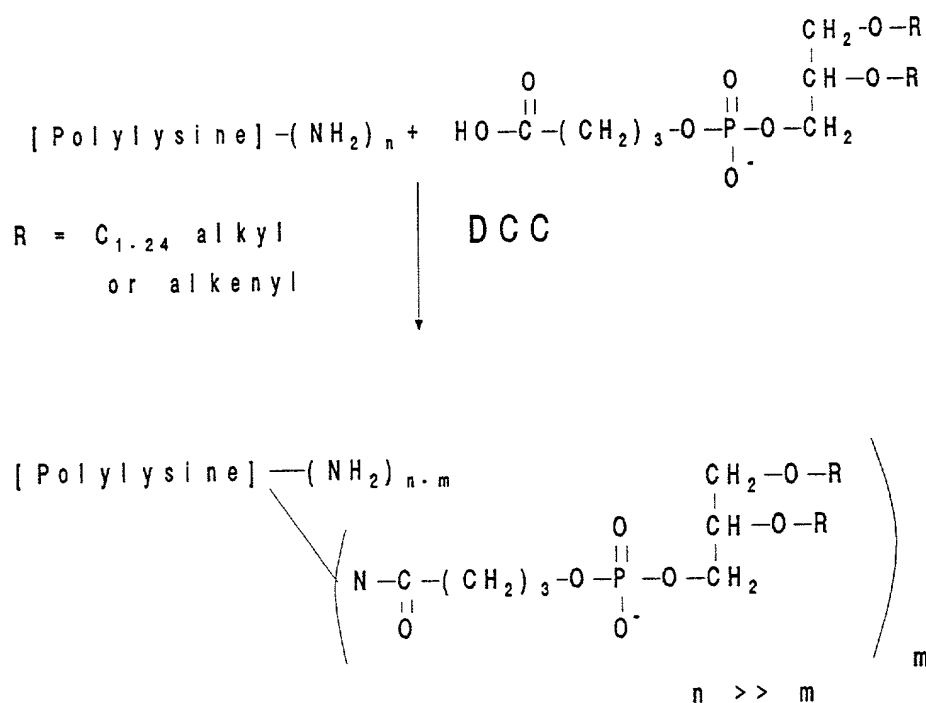


R = C<sub>1-24</sub> alkyl, alkenyl

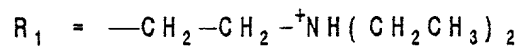
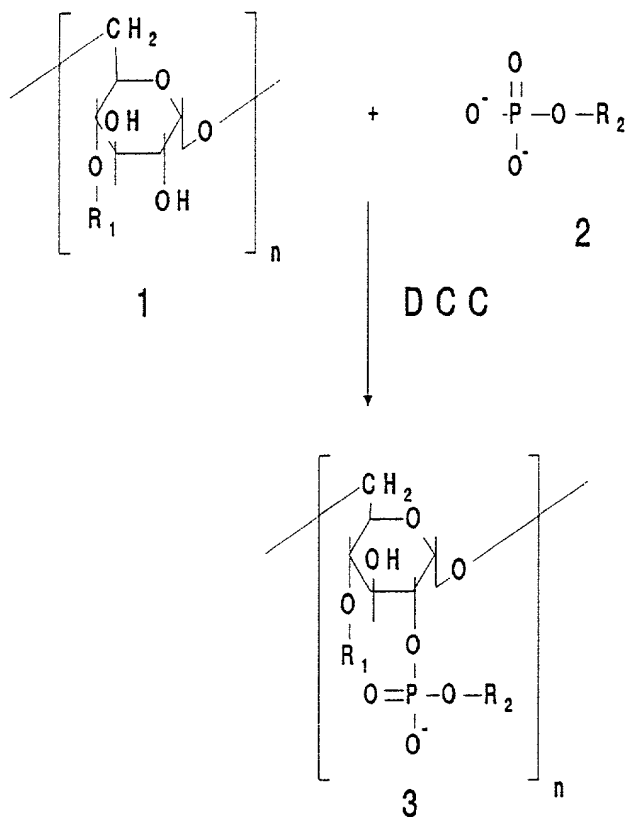
SCHEME 10 (continued)



SCHEME 11



SCHEME 12

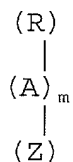


$\text{R}_2$  = straight chain or branched alkyl, alkenyl, cycloalkyl, aryl, alkoxy, thioalkyl or thioether group having from 12 to about 24 carbon atoms

$n$  = 50 - 600 (chain length)

WE CLAIM:

1. A compound having the structure:



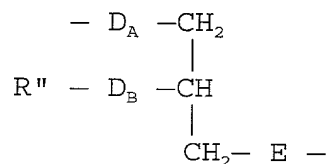
and salts thereof where:

R is  $R_A$  or  $R_B$ , where  $R_A$  is  $C_{1-23}$  alkyl or alkenyl and  $R_B$  is a steroid selected from the group consisting of stigmasterol, ergosterol and cholic acid;

m is 0 or 1, where m is 0 when Z is  $Z_{16}$  or  $Z_{17}$  and m is 1 when Z is  $Z_1$ - $Z_{15}$  and  $Z_{18}$ ;

A is selected from any of  $A_1$ - $A_3$  where:

$A_1$  is



where  $D_A$  is bonded to R and E is bonded to Z;

$R''$ , independently of R, is  $R_A$  or  $R_B$ , where  $R_A$  is  $C_{1-23}$  alkyl or alkenyl and  $R_B$  is a steroid selected from the group consisting of stigmasterol, ergosterol and cholic acid;

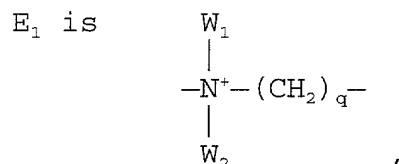
$D_A$  and  $D_B$ , independently of one another, are selected from the group consisting of  $D_1$ - $D_3$  where:

$D_1$  is  $-Y_1-CO-Y_2-$ , where  $Y_1$  and  $Y_2$ , independently of one another, are O and NH, and wherein at least one of  $Y_1$  and  $Y_2$  is NH;

$D_2$  is  $-CH=CH-O-$ ; and

$D_3$  is  $-O-$  or  $-CO_2-$ ;

E is selected from the group consisting of E<sub>1</sub>-E<sub>3</sub>, where



where W<sub>1</sub> and W<sub>2</sub>, independently of one another, are C<sub>1-24</sub> alkyl, alkenyl or aryl; q is 1 to 6;

E<sub>2</sub> is -PO<sub>4</sub><sup>-</sup>-(CH<sub>2</sub>)<sub>2</sub>-NH-; and

E<sub>3</sub> is -(PO<sub>4</sub><sup>-</sup>)<sub>r</sub>-[inositol]-NH-, where r is 1 or 2;



where B<sub>A</sub>-B<sub>C</sub>, independently of one another, are selected from the group consisting of the following groups B<sub>1</sub>-B<sub>4</sub>, wherein at least one of B<sub>A</sub>-B<sub>C</sub> is B<sub>1</sub>, at least one of B<sub>A</sub>-B<sub>C</sub> is B<sub>2</sub>, and at least one of B<sub>A</sub>-B<sub>C</sub> is B<sub>3</sub> or B<sub>4</sub>, where:

B<sub>1</sub> is -OH;

B<sub>2</sub> is -NH-R, where R is C<sub>1-23</sub> alkyl, alkenyl or acyl;

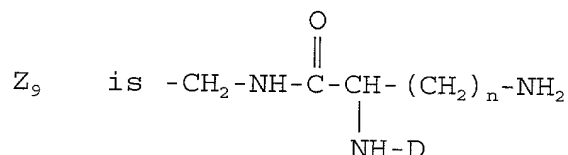
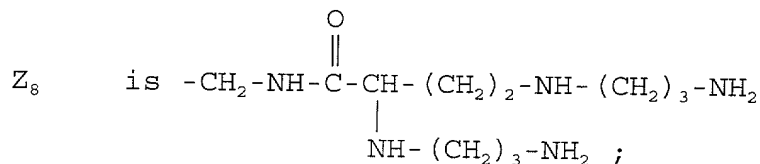
B<sub>3</sub> is -O-Z or -NH-Z; and

B<sub>4</sub> is -PO<sub>4</sub><sup>-</sup>-(CH<sub>2</sub>)<sub>2</sub>-NH-Z; and

A<sub>3</sub> is -NH-CH<sub>2</sub>- or -CO-N-R<sub>1</sub>-, where A is -NH-CH<sub>2</sub>- when R is cholic acid and A is -CO-N-R<sub>1</sub>- when R is stigmasterol or ergosterol; where R<sub>1</sub> is an alkyl, alkenyl, alkynyl, alkoxy, acyl or alkylthio having from 1 to about 24 carbon atoms; and

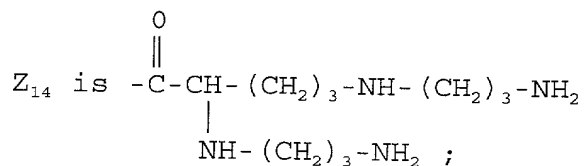
where Z is selected from the group consisting of  $Z_1$ - $Z_{15}$  or  $Z_{18}$ ; where:

- $Z_1$  is H except where  $W_1$  and  $W_2$  are methyl;  
 $Z_2$  is  $-(CH_2)_n-X$ , where n is 1-24 and X is selected from the group consisting of Br, Cl, I and F;  
 $Z_3$  is  $-(CH_2)_n-NH_2$ , n = 1-24;  
 $Z_4$  is  $-CH_2-NH-(CH_2)_3-NH-(CH_2)_4-NH_2$ ;  
 $Z_5$  is  $-CH_2-NH-(CH_2)_3-NH-(CH_2)_3-NH-(CH_2)_3-NH_2$ ;  
 $Z_6$  is  $-CH_2-NH-(CH_2)_n-NH_2$ , n = 2-24;  
 $Z_7$  is  $-L-X$  where L is selected from the group consisting of branched or straight chain alkyl, alkenyl, cycloalkyl, aryl, alkoxy, thioalkyl and thioether groups having from 1 to about 24 carbon atoms, and X is selected from the group consisting of Br, Cl, I, F,  $NH_2$  and  $[(NH_2)-(CH_2)_n]_m$  where n is 2-24 and m is 1-24;

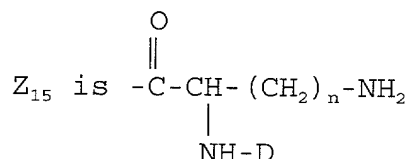


where n = 1-24, D is H or other groups attached by amide or alkyl amino groups;

- $Z_{10}$  is a reporter molecule;  
 $Z_{11}$  is a protein, peptide or polypeptide;  
 $Z_{12}$  is a polysaccharide;  
 $Z_{13}$  is an amine or halide reactive group;





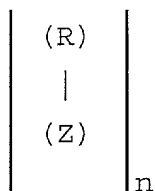


$n = 1-24$ , D is H or other groups attached by amide or alkyl amino groups; and

$Z_{18}$  is a nucleic acid binding substance.

2. The compound of claim 1 wherein A is  $A_3$ .
3. The compound of claim 2 wherein  $A_3$  is  $-\text{CO}-\text{N}-\text{R}_1-$  and  $\text{R}_B$  is stigmasterol.
4. The compound of claim 2 wherein  $A_3$  is  $-\text{CO}-\text{N}-\text{R}_1-$  and  $\text{R}_B$  is ergosterol.
5. The compound of claim 1 wherein D is  $D_3$ .
6. The compound of claim 5 wherein R is  $\text{R}_A$ , A is  $A_1$  and E is  $E_3$ .
7. A composition for transfecting a cell with a nucleic acid which comprise a nucleic acid and one or more compounds according to claim 1.
8. A lipid aggregate which comprises one or more compounds of claim 1.
9. A kit for preparing a lipid aggregate comprising one or more cationic lipids according to claim 1.
10. A method for transfecting a cell comprising the step of contacting the cell with a lipid aggregate comprising a nucleic acid and a compound according to claim 1.

11. A compound having the repeating structure:



and salts thereof where:

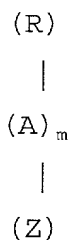
n is an integer ranging from 2 to about 2,000;

R is R<sub>A</sub> or R<sub>B</sub>, where R<sub>A</sub> is C<sub>1-23</sub> alkyl or alkenyl and R<sub>B</sub> is a steroid selected from the group consisting of stigmasterol, ergosterol and cholic acid; and

Z is selected from the group consisting of an amino acid and a monosaccharide.

12. A compound according to claim 11 where Z is a basic amino acid.
13. A compound according to claim 12 where Z is selected from the group of amino acids consisting of ornithine, lysine, arginine and histidine.
14. A compound according to claim 12 where Z is L-ornithine.
15. A compound according to claim 11 where Z is a sugar having from 3 to about 7 carbon atoms.
16. A compound according to claim 15 where said sugar comprises a cationic substituent.
17. A compound according to claim 16 where said cationic substituent is a tertiary amine.
18. A compound according to claim 16 where said cationic substituent is diethylaminoethyl.

19. A compound according to claim 16 where said sugar is glucose and said cationic substituent is diethylaminoethyl.
20. A composition for transfecting a cell with a nucleic acid which comprise a nucleic acid and one or more compounds according to claim 11.
21. A lipid aggregate which comprises one or more compounds of claim 11.
22. A method for transfecting a cell comprising the step of contacting the cell with a lipid aggregate comprising a nucleic acid and one or more compounds according to claim 11.
23. A method for transfecting a cell comprising the step of contacting the cell with a lipid aggregate comprising a nucleic acid and one or more cationic lipids having the structure:



and salts thereof where:

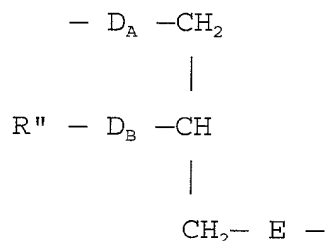
R is  $R_A$  or  $R_B$ , where  $R_A$  is  $C_{1-23}$  alkyl or alkenyl and  $R_B$  is a steroid selected from the group consisting of stigmasterol, ergosterol and cholic acid;

m is 0 or 1, where m is 0 when Z is  $Z_{16}$  or  $Z_{17}$  and m is 1

when Z is  $Z_1$ - $Z_{15}$  and  $Z_{18}$ ;

A is selected from any of  $A_1$ - $A_2$  where:

$A_1$  is



where  $D_A$  is bonded to R and E is bonded to Z;

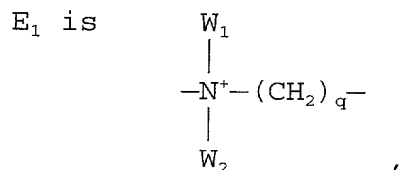
$R''$ , independently of R, is  $R_A$  or  $R_B$ , where  $R_A$  is  $C_{1-23}$  alkyl or alkenyl and  $R_B$  is a steroid selected from the group consisting of stigmasterol, ergosterol and cholic acid;

$D_A$  and  $D_B$ , independently of one another, are selected from the group consisting of  $D_1$ - $D_2$  where:

$D_1$  is  $-Y_1-CO-Y_2-$ , where  $Y_1$  and  $Y_2$ , independently of one another, are O and NH, and wherein at least one of  $Y_1$  and  $Y_2$  is NH; and

$D_2$  is  $-CH=CH-O-$ ;

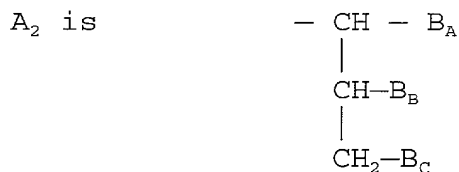
E is selected from the group consisting of  $E_1$ - $E_3$  where



where  $W_1$  and  $W_2$ , independently of one another, are  $C_{1-24}$  alkyl, alkenyl or aryl; q is 1 to 6;

E<sub>2</sub> is  $-\text{PO}_4^--(\text{CH}_2)_2-\text{NH}-$ ; and

E<sub>3</sub> is  $-(\text{PO}_4^-)_r-[\text{inositol}]-\text{NH}-$ , where r is 1 or 2; and



where B<sub>A</sub>-B<sub>C</sub>, independently of one another, are selected from the group consisting of the following groups B<sub>1</sub>-B<sub>4</sub>, wherein at least one of B<sub>A</sub>-B<sub>C</sub> is B<sub>1</sub>, at least one of B<sub>A</sub>-B<sub>C</sub> is B<sub>2</sub>, and at least one of B<sub>A</sub>-B<sub>C</sub> is B<sub>3</sub> or B<sub>4</sub>, where:

B<sub>1</sub> is  $-\text{OH}$ ;

B<sub>2</sub> is  $-\text{NH}-\text{R}$ , where R is C<sub>1-23</sub> alkyl, alkenyl or acyl;

B<sub>3</sub> is  $-\text{O}-\text{Z}$  or  $-\text{NH}-\text{Z}$ ; and

B<sub>4</sub> is  $-\text{PO}_4^--(\text{CH}_2)_2-\text{NH}-\text{Z}$ ; and

where Z is selected from the group consisting of Z<sub>1</sub>-Z<sub>15</sub> or Z<sub>18</sub>; where:

Z<sub>1</sub> is H except where W<sub>1</sub> and W<sub>2</sub> are methyl;

Z<sub>2</sub> is  $-(\text{CH}_2)_n-\text{X}$ , where n is 1-24 and X is selected from the group consisting of Br, Cl, I and F;

Z<sub>3</sub> is  $-(\text{CH}_2)_n-\text{NH}_2$ , n = 1-24;

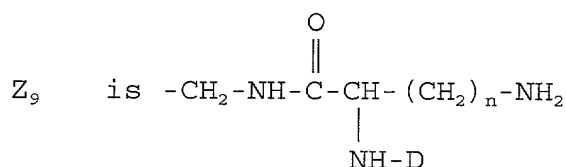
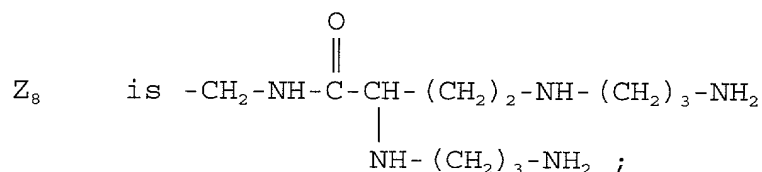
Z<sub>4</sub> is  $-\text{CH}_2-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$ ;

Z<sub>5</sub> is  $-\text{CH}_2-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$ ;

Z<sub>6</sub> is  $-\text{CH}_2-\text{NH}-(\text{CH}_2)_n-\text{NH}_2$ , n = 2-24;

Z<sub>7</sub> is  $-\text{L}-\text{X}$  where L is selected from the group consisting of branched or straight chain alkyl,

alkenyl, cycloalkyl, aryl, alkoxy, thioalkyl and thioether groups having from 1 to about 24 carbon atoms, and X is selected from the group consisting of Br, Cl, I, F, NH<sub>2</sub> and [(NH<sub>2</sub>)-(CH<sub>2</sub>)<sub>n</sub>]<sub>m</sub> where n is 2-24 and m is 1-24;



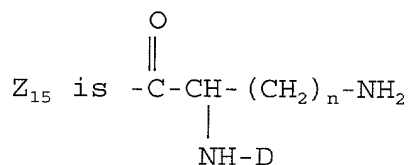
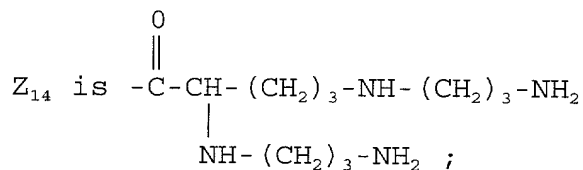
where n = 1-24, D is H or other groups attached by amide or alkyl amino groups;

Z<sub>10</sub> is a reporter molecule;

Z<sub>11</sub> is a protein, peptide or polypeptide;

Z<sub>12</sub> is a polysaccharide;

Z<sub>13</sub> is an amine or halide reactive group;



where n = 1-24, D is H or other groups attached by amide or alkyl amino groups; and

Z<sub>18</sub> is a nucleic acid binding substance.

24. The method of claim 23 wherein in the cationic lipid when A is A<sub>2</sub>, Z is Z<sub>14</sub>.
25. A composition for transfecting a cell with a nucleic acid which comprises a cationic lipid of claim 1 capable of complexing said nucleic acid to be transfected into said cell, and a transfection-enhancing agent selected from the group consisting of an enveloped virus, a membrane virus, a viral component, and a non-viral fusagenic compound.
26. A composition according to claim 25 wherein said transfection-enhancing agent is an enveloped virus, and wherein said enveloped virus is an alphavirus.
27. A composition according to claim 26 wherein said alphavirus is Semliki Forest virus.
28. A composition according to claim 25 wherein said transfection-enhancing agent is a viral component and wherein said viral component is selected from the group consisting of viral proteins, envelope fusion peptides, viral spike glycoproteins, viral peptides of viral spike glycoproteins, and viral envelope fragments containing embedded viral protein.
29. A composition according to claim 25 wherein said transfection-enhancing agent is a non-viral fusagenic peptide.
30. A method for transfecting a cell comprising the steps of contacting the cell with a transfecting composition of claim 25.

## ABSTRACT OF THE INVENTION

The present invention provides certain cationic lipids containing stigmasterol, ergosterol and cholic acid groups. Compounds of the invention are useful, either alone or in combination with other lipid aggregate-forming components (e.g., DOPE, DOPPA, DOTMA or cholesterol) for formulation into liposomes or other lipid aggregates. Such aggregates are cationic, and able to form stable complexes with anionic macromolecules, such as nucleic acids. The cationic lipids of the invention are useful in methods of transfecting cells, particularly to introduce nucleic acids into cells. The invention also related to kits for the preparation of lipid aggregates and to lipid aggregates and compositions for transfection of cells.



# JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated below our names;

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled "NOVEL REAGENTS FOR INTRACELLULAR DELIVERY OF MACROMOLECULES", specification of which was filed on February 11, 1994 as Application Serial No. 08/195,866.

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by an amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed;

## Prior Foreign Application(s)

Country	Application No.	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed Under 35 U.S.C. 119
NONE				Yes___ No___ Yes___ No___ Yes___ No___ Yes___ No___

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application;

Application Serial Number	Date of Filing (day, month, year)	Status--Patented Pending, Abandoned
NONE		

And we hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys, their registration numbers being listed after their names:

Lorance L. Greenlee, Reg. No. 27,894; Ellen P. Winner, Reg. No. 28,547; Sally A. Sullivan, Reg. No. 32,064; Donna M. Ferber, Reg. No. 33,878; Jennie M. Caruthers, Reg. No. 34,464; Barbara A. Gyure, Reg. No. 34,614; Charles E. Rohrer, Reg. No. 24,085; and Alison A. Langford, Reg. No. 37,374.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature Gulilat Gebeyehu Date 3/4/94

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